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ELECTROPHORESIS AND RELATED METHODS

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REVIEW

# STARCH ELECTROPHORESIS

## II. STARCH COLUMN ELECTROPHORESIS

H. BLOEMENDAL

*Department of Biochemistry, Antoni van Leeuwenhoek-Huis:*
*The Netherlands Cancer Institute, Amsterdam (The Netherlands)*

(Received June 5th, 1959)

In the first part of this survey on starch electrophoresis[63] the starch block technique was discussed and the conclusion was drawn that generally the best separations are realised when the starch block is in a vertical position during the electrophoretic run. In principle similar results are obtained when columns are used. Nevertheless I wish to make a distinction between vertical starch block electrophoresis and starch column electrophoresis, because the analytical operations [illegible] the experiment are different in these two methods. In the first technique the block is cut into equal segments to separate the zones, whereas in the second the separated zones are forced out by means of a buffer stream, which makes the use of a fraction collector necessary. Although originally only starch was used as the inert supporting medium[3,4] in column electrophoresis, at present there is a tendency to apply cellulose powders instead of starch[64-69]. Actually more separations are carried out with other media than starch as stationary phase. These experiments will not be discussed in this article. But even cellulose column electrophoresis is not so widely applied as starch block electrophoresis and starch gel electrophoresis. Undoubtedly the reason for this is that in general no better results are obtained with column electrophoresis than with the two last-mentioned techniques, while its procedure is more complicated.

### METHODS

### 1. *Apparatus*

**Haglund and Tiselius**[70] described an apparatus for glass powder electrophoresis, which consisted mainly of a U tube and two electrode vessels. This apparatus was improved by **Flodin and Porath**[4] who replaced the glass powder by potato starch.

A glass tube s (Fig. 7), 50 × 3 cm, with a sintered glass filter f at the bottom is joined to a second tube by means of rubber tubing. The porosity and thickness of the glass filter must be chosen so that the electrical resistance is as small as possible. The filter surface may be covered with a disc of Whatman No. 1 paper. The tube, which is filled with starch, is connected to the cathode and the second tube is connected to the anode, both connections being made with rubber tubing that can be closed by clamps. Reversible silver–silver chloride electrodes as well as platinum wire wound round glass rods may be used as cathode and anode. **Haglund and Tiselius** recommend the use of an open system, which allows the electroosmotic flow to develop freely. This is realized by connecting a narrow horizontal glass tube, open at one point, to the electrode vessels. In this way a constant hydrostatic pressure is maintained in the whole system during the electrophoretic run. In some experiments the apparatus is placed in a thermostat.

Vandegaer, Préaux and Lontie[71] found a more simple solution for the cooling problem. The apparatus that these investigators employed was in principle the same as that of Flodin and Porath, but they constructed a water-jacketed column. Efficient cooling was obtained by tap water of 2°.

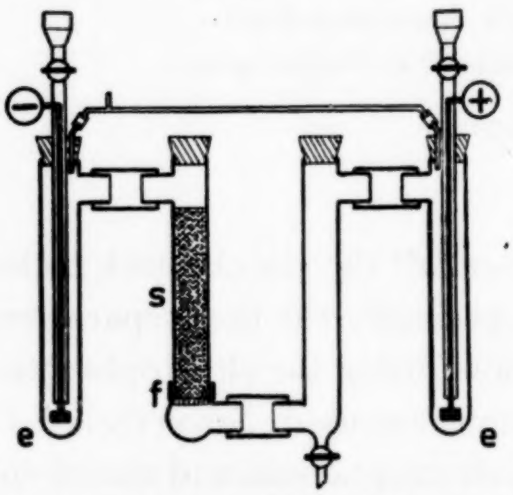

Fig. 7. Apparatus according to Haglund and Tiselius. e = electrodes; s = tube filled with starch; f = glass filter.

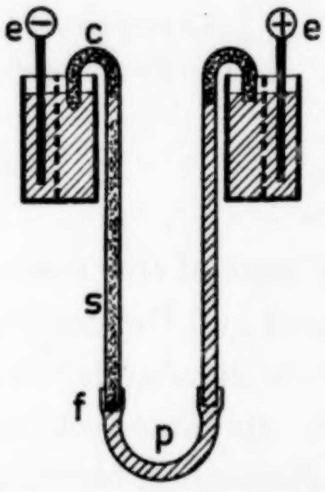

Fig. 8. Apparatus according to Kunkel. e = electrodes; s = tube filled with starch; f = filter disc; c = cotton wicks; p = plastic tubing.

Carlson[3], keeping to the design of Haglund and Tiselius, placed a central cooling tube in the starch column.

Bolinger, Van der Geld, Willebrands and Groen[72] improved the original apparatus of Flodin and Porath by applying a device that allowed constant removal of the electrolysis products from the electrode vessels.

Kunkel[73] described an apparatus that can easily be constructed in any laboratory. Two glass tubes s, 40 × 1 cm, are joined together by rubber or plastic tubing p (Fig. 8). Tube s is fitted with a glass filter f to support the starch. The connections with the electrode vessels consist of cotton wicks c enclosed in bent glass tubes or merely covered with cellophane or parafilm.

Svensson[74], in collaboration with Flodin and Porath, designed a commercial apparatus. The glass tube is surrounded by a water jacket, and the connections with the electrode vessels and the special filling device are supplied with ground glass joints. For preparative work, columns of considerable length may be used. Porath[69] purified posterior pituitary extracts in externally cooled columns of 300 × 1.5 cm. These experiments were carried out on cellulose powder, but starch columns of comparable or even greater length may be applied.

## 2. *Preparation of starch*

Homogeneous packing is essential in all chromatographic techniques where powdered materials are used. Also, in starch column electrophoresis irregular filling of the tube would result in tailing and spreading of the zones. The starch may conveniently be prepared as described in Part I[63]. FLODIN AND PORATH recommend the use of a starch fraction that passes a 300-mesh sieve. Application of the packing method of MOORE AND STEIN[75] also gives good results. In this technique starch is suspended in butanol and ground in a mortar. Distilled water is added to the suspension until the amount is 30% of the dry starch. The slurry is poured into the column and slight air pressure is applied. The starch is allowed to settle during a period of one hour. Air bubbles can be avoided by dipping the empty column in water so that the filter-disc surface (Fig. 7, f) within the glass tube is covered with the liquid. The butanol above the starch is withdrawn by suction. Afterwards water is poured in very carefully and the column is connected to a Mariotte flask filled with distilled water. The water is allowed to pass through the column until an eluate shows no, or minimal absorption at 260 or 280 m$\mu$ against a water control. These washings are repeated with the buffer solution in which the electrophoresis will be carried out. The column must be kept exactly vertical during the electrophoretic run as well as during the elution.

### 3. *Introduction of the sample*

Before the electrophoretic run is started, the buffer solution above the starch surface is withdrawn with a pipette or driven down by slight pressure so that the upper starch layer is just dry. The sample is introduced dropwise by means of a funnel fitted with a narrow tube or simply with a pipette. The test solution is then slowly forced into the column by slight pressure. If there are components that will migrate towards the cathode, the sample must be transported over a certain distance before electrophoresis is started. In order to determine this distance the most convenient method is to add known amounts of buffer solution. The relation between the required buffer volume and the vertical displacement is easily found by trial using a coloured substance (*e.g.* haemoglobin or albumin stained with bromophenol blue). When the sample is in the desired position, the column is filled with buffer and connected to the electrodes.

### 4. *Electrophoresis*

As in the case of starch block electrophoresis, there are no standardized conditions for all starch column electrophoresis experiments. The most favourable voltage, current, buffer, and the duration of the electrophoretic run must be found by trial. Much depends on the packing and the length of the column. Though continuous cooling by circulating ice water allows the application of higher voltages than those used without cooling, the heat development even then represents a serious limitation of the method. In many cases asymmetric band formation causes insufficient separation. This effect becomes serious when columns with a large diameter are used. The best results are generally obtained with columns not much longer than 1 m and with a diameter not exceeding 6 cm.

CARLSON[3] observed considerable spreading of the zones when phosphate buffers were used. Veronal and ammonium buffers gave better results. Increasing the salt concentration had no influence upon the spreading of the protein zones.

According to **Flodin and Kupke**[67] citrate and phosphate buffers give rise to strong electroosmotic flow in the starch medium.

In Table III the working conditions of a few investigators are summarized.

TABLE III

| *Material* | *Buffer* | *Ionic strength* | *pH* | *V* | *mA* | *h* | *Reference* |
|---|---|---|---|---|---|---|---|
| Serum lipoproteins | ammonium | 0.1 | 9 | 350 | — | — | 3 |
| Serum | veronal | 0.05 | 8.6 | 700 | 25 | 12–20 | 4 |
| Bacitracine polypeptide | acetate | 0.15 | 4.5 | — | 35 | 44 | 4 |
| Serum | veronal | 0.1 | 8.6 | 400 | 5 | 18 | 14 |
| Insulin activity in serum | bicarbonate | — | 8.0–8.3<br>6.8–7.0 | 350 | 100 | 24 | 72 |
| Thiamine and its phosphoric esters | phosphate | 0.05 | 5.44 | — | 17 | 12 | 82 |
| Vitamin $B_6$ compounds | acetate | 0.05 | 5.1 | — | 18 | 14 | 83 |
| Pancreatic lipase | acetate* | 0.025 | 5.25 | 8 V/cm | — | 48 | 85 |

* 0.005 $M$ $CaCl_2$ added.

### 5. *Influence of temperature*

As mentioned earlier the rise of temperature as a result of the application of high voltages represents one of the most serious limitations of starch electrophoresis. In practice the relation between the applied voltage and the rise of temperature during the electrophoretic run is easily determined by measuring the temperature in the column with a thermistor at certain intervals and plotting the temperature against the time (*cf.* Fig. 3 in Part I). Only a little theoretical work has been reported on the problem of temperature distribution in columns[76,77]. Recently **Porath**[78] described a device for temperature measurements in cylindrical electrophoresis columns without central cooling. This author derived a fairly simple equation which allows a rough estimation of the permissible current values in an electrophoresis experiment.

The assumption is made that heat is transported only by conduction. In this case the heat flow at time $t$ at any point of the system is described by the general equation:

$$\frac{\partial T}{\partial t} = \operatorname{div} \frac{\lambda}{c\rho} \operatorname{grad} T + \frac{Q}{c\rho} \qquad (1)$$

in which $T$ = temperature
$\lambda$ = thermal conductivity coefficient
$c$ = specific heat
$\rho$ = density
$Q$ = heat generated per unit time and volume.

For cylindrical columns packed with powders like starch this equation reduces to:

$$c\rho \frac{\partial T}{\partial t} = \lambda \Delta T + Q \qquad (2)$$

in which $\Delta$ = Laplacian operator.

It can be deduced that if the internally and externally cooled column were filled with buffer alone, the heat generated would be:

$$Q = 0.239 \frac{i^2}{\varkappa} \tag{3}$$

in which $i$ = current density
$\varkappa$ = conductivity of the buffer solution
$Q$ = heat generated (expressed in calories per $cm^3$).

Actually the effective cross section is reduced by the uncharged starch particles so that equation (3) becomes:

$$Q = 0.239\, k \frac{I^2}{q^2 \varkappa} \tag{4}$$

in which $k$ = a constant characteristic for the (starch) medium
$I$ = current
$q$ = cross section of the column.

According to PORATH the water content in starch and cellulose differs considerably (33 and 85 % per volume, respectively). This difference is reflected in the $k$ values.

Furthermore, PORATH evolved an equation which holds when the column is cooled with circulating water of constant temperature without central cooling:

$$T_m - T_r = \frac{Qr^2}{4\,\lambda_c} \tag{5}$$

in which $T_m$ = maximum temperature
$T_r$ = temperature at $r$ cm from the axis
$\lambda_c$ = conductivity coefficient of the column content.

($\lambda_c = 2.2 \cdot 10^{-3}$ cal·degree $C^{-1} \cdot cm^{-1} \cdot sec^{-1}$ for starch packed in a column of 105 cm length and 3.4 cm diameter).

Inserting $Q$ from equations (4) and (5) gives:

$$T_m - T_r = 0.239\, k \frac{I^2 r^2}{4\, q^2 \varkappa \lambda_c} \tag{6a}$$

$$\frac{0.239\, k}{4\, \pi^2 \lambda_c} = K$$

$$T_m - T_r = \frac{K \cdot I^2 \cdot r^2}{R^4 \varkappa} \tag{6b}$$

in which $R$ = inner radius of the column.

Fig. 9 is the graphic representation of the latter equation, in which $K/\varkappa = 3200$, $I_{\mathrm{I}} = 150$ mA, $I_{\mathrm{II}} = 90$ mA.

For the difference between the temperature at the center $T_m$ and the periphery $T_0$ (6b) simplifies to:

$$T_m - T_0 = \frac{K \cdot I^2}{R^2 \varkappa} \tag{7}$$

**Porath** found that $k$ has the value 4.12 in starch columns (determined at 15° for starch–0.1 $M$ pyridinium acetate medium).

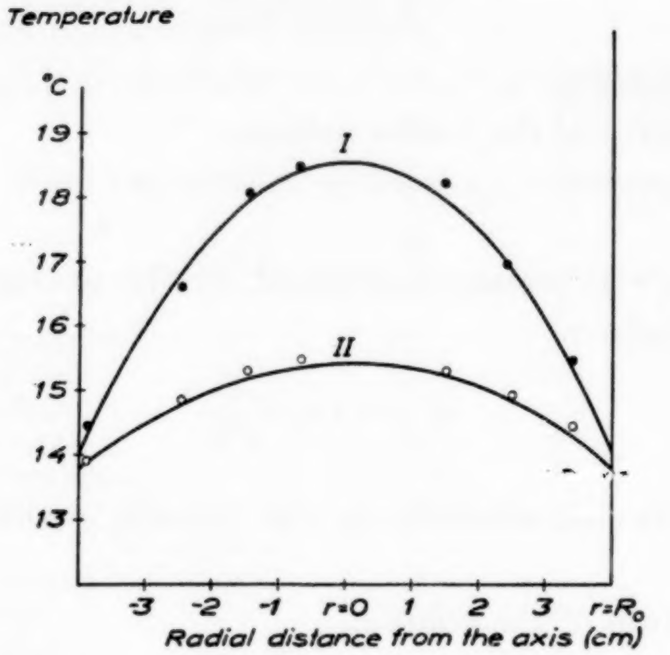

Fig. 9. Radial temperature distribution in a starch column 105 × 3.4 cm, 12 cm below the starch surface; current in I, 150 mA; current in II, 90 mA. (Reprinted with the permission of Dr. J. Porath.)

## 6. *Elution and automatic analysis*

After the electrophoretic run the electrode vessels are disconnected and removed, and a Mariotte flask filled with elution liquid is connected to the column by means of rubber or plastic tubing. The Mariotte flask is used in order to obtain a constant hydrostatic pressure during elution. It is possible to collect the separated components in reagent tubes manually, but this is a very time-consuming business, so that an automatic fraction collector becomes really indispensable. Further saving of time is obtained by analysing the eluates in a continuous analyser.

In our laboratory a very simple apparatus has been constructed, which allows

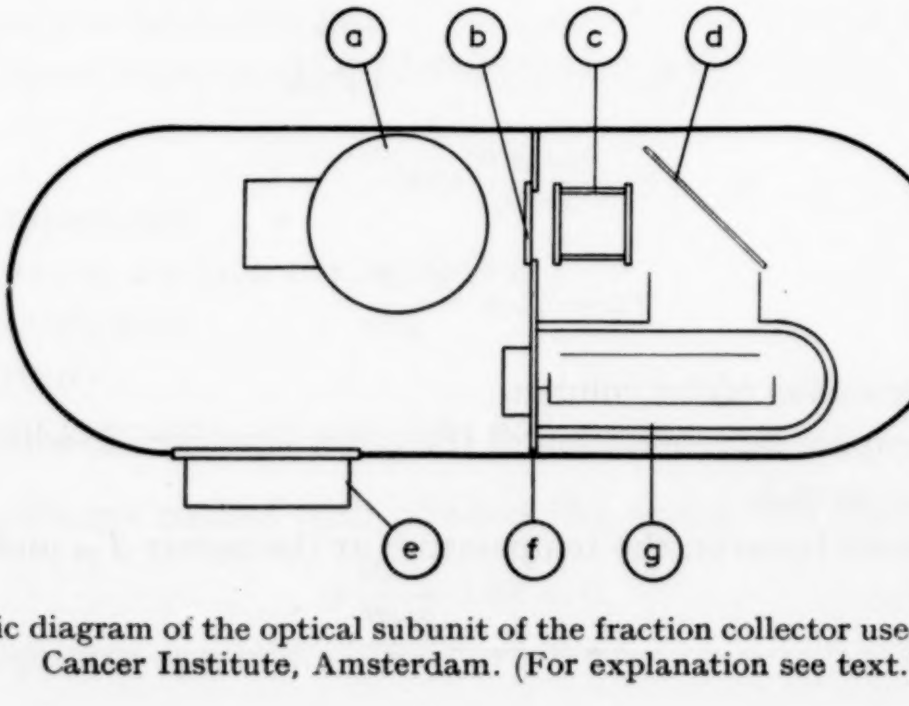

Fig. 10. Schematic diagram of the optical subunit of the fraction collector used in The Netherlands Cancer Institute, Amsterdam. (For explanation see text.)

continuous estimation of nucleotides, polynucleotides, and proteins[79] (Fig. 10). The light source is a low-pressure mercury lamp, a. The lamp compartment is divided into two parts by means of the partition f, in which there is a quartz window, b. The ultraviolet light-beam falls through the glass cuvette c, which is likewise supplied with a quartz window. The cuvette has been constructed in such a way that air bubbles can pass through without adhering to the wall. The transmitted light is not measured directly but via the fluorescent screen, d. The rejected visible light is measured with a common photocell, g. If it is desired to measure at other wave lengths the mercury lamp can be replaced by other suitable light sources, if necessary in combination with filters. The optical subunit is connected to a recorder via a multipole plug, e. The fraction collector (Fig. 11) is furnished with a volume-measuring system. At the very moment that a certain tube has been filled, the connection between the vacuum tube volt meter and recorder is interrupted for a short time. The pen-recorder then falls back to its zero position. In this way, besides the registered curve, straight lines are drawn on the paper indicating the transition from one tube to the following one*.

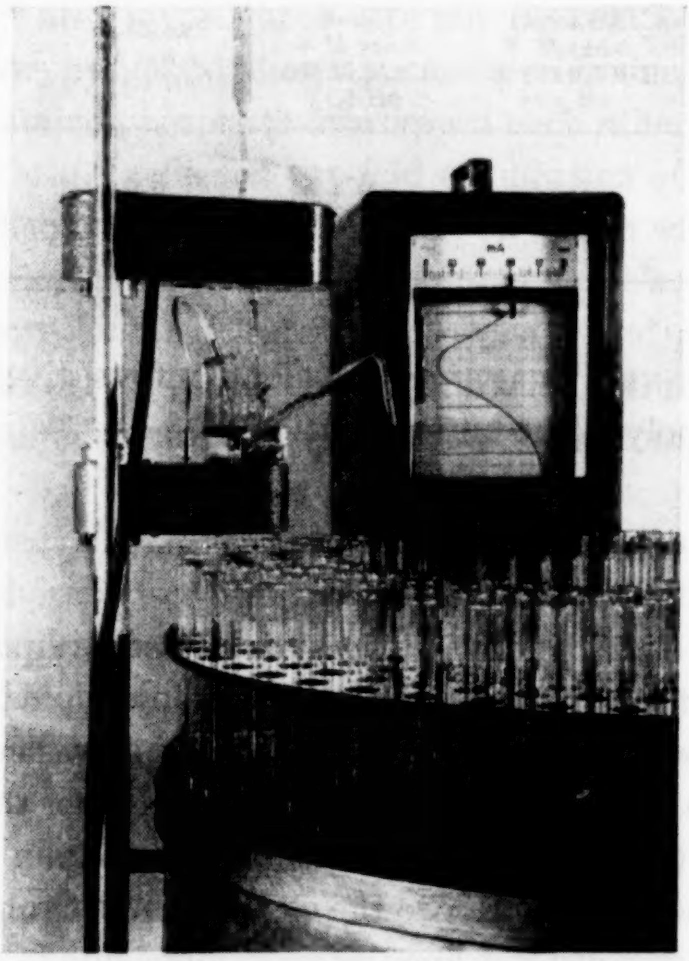

Fig. 11. Automatic fraction collector with recorder.

PORATH and coworkers[87] designed an apparatus which allows continuous removal of the separated zones during electrophoresis. A buffer flow is applied in the opposite direction of the electrophoretic migration. This device permits the use of short columns and seems to counteract the broadening of zones during elution.

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* Similar fraction collectors are commercially available (Dr. Gilson, Madison, Wisconsin, U.S.A.).

### 7. *Influence of electroosmosis*

As described in part I[63] the electroosmotic flow is detected with the aid of substances such as dextran or glucose that do not migrate in the electric field. However, it must be taken into account that there is some interaction between starch and glucose, so corrections must be made for the resulting retardation effect on the glucose molecules. MARCHIS-MOUREN *et al.*[85] gave examples of the electroosmotic flow under various conditions (Table IV).

TABLE IV

| *Buffer* | *Na acetate 0.025 M + $CaCl_2$ 0.005 M pH 5.25* | *Citrate 0.025 M + $CaCl_2$ M/600 pH 6.15* | *Phosphate 0.025 M pH 6.75* | *Veronal 0.025 M + $CaCl_2$ 0.005 M pH 8.0* | *Veronal 0.05 M + $CaCl_2$ 0.005 M pH 8.0* |
|---|---|---|---|---|---|
| Potential gradient (V/cm) | 6.15 | 9.4 | 8.0 | 6.7 | 6.7 |
| Flow (cm/h) | — 0.64 | — 1.1 | — 0.78 | — 0.51 | — 0.76 |

According to FLODIN AND PORATH[4] monovalent buffers give rise to lower electroosmotic flow than do polyvalent buffer solutions.

## APPLICATIONS

CARLSON[3] studied the electrophoretic behaviour of serum lipoproteins in nephrosis, diabetes, and essential hyperlipaemia. The recovery of the lipids and protein was about 100 % in monovalent buffers such as veronal. Adsorption was noticed when phosphate buffer was used. When the protein concentration was higher than 2%, spreading and overlapping of zones took place. The migration of albumin could be followed by staining with bromophenol blue prior to the electrophoretic run. The method revealed that there are at least five distinct lipoprotein components in serum.

CARLSON AND OLHAGEN[80] investigated chylomicrons in a case of essential hyperlipaemia. The chylomicron migrated as two distinct fractions. One fraction was associated with α-globulin, the other with β-globulin. These findings were in agreement with the results obtained with free electrophoresis, whereas paper electrophoresis gave only a single fraction[81].

FLODIN AND PORATH[4] separated human serum proteins and obtained patterns that agreed very well with the results obtained with other zone electrophoresis techniques. The same authors submitted an antibiotic mixture containing bacitracin to column electrophoresis on starch. Two main peaks were found. The bulk of the activity appeared to be concentrated in the largest peak. Other methods, however, revealed heterogeneity of this peak.

KUNKEL[73] fractionated serum. In some experiments the sample was made to migrate up the column; about 100 % recovery was obtained.

BOLINGER and coworkers[72] separated normal human serum. The fractions obtained were tested for insulin activity and the main activity was found in the β-globulin fraction. $^{131}$I was detected in the same fraction when it was added to the serum. Furthermore, some minor activity was found in the $\gamma$-globulins, indicating that in serum endogenous insulin is linked to different proteins.

SILIPRANDI AND SILIPRANDI[82] separated thiamine and its phosphate esters. Thiamine and its monophosphate moved towards the cathode, whereas diphospothiamine and triphosphothiamine migrated towards the anode. However, cellulose powder as inert supporting medium appeared to be superior. With this medium complete separation of thiamine and its phosphoric esters could be realised and quantitative recovery of each compound was achieved.

SILIPRANDI *et al.*[83] also investigated vitamin $B_6$ compounds. Resolution of a mixture of pyridoxamine, pyridoxal phosphate and pyridoxine was observed, whereas pyridoxal and pyridoxamine phosphate overlapped each other partially.

MARCHIS-MOUREN *et al.*[85] achieved 135-fold purification of pancreatic lipase with a 20 % overall yield, when column electrophoresis on starch was used as final step in a fractionation scheme including ammonium sulphate and acetone precipitation as well as differential adsorption on tricalcium phosphate and aluminium hydroxide. A higher potential gradient (8 V/cm), longer duration of the electrophoretic run and the use of an acid buffer resulted in higher resolution of components than reported earlier[84].

BOUSSIER[86] obtained a pure compound of serum mucoprotein haptoglobin. Starch column electrophoresis was applied in combination with ammonium sulphate fractionation and starch gel electrophoresis.

The last-mentioned technique, which is of growing importance and may possibly supersede paper electrophoresis, will be discussed in the last part of this review.

## CONCLUSION

Starch column electrophoresis is not used so widely as the simpler block method. A disadvantage of the former technique is the necessity of elution by means of a buffer flow after the electrophoretic run, which gives rise to broadening of the zones. According to KUNKEL AND TRAUTMAN[88] as much as a two-fold increase in the width of a haemoglobin band was found when elution over a fairly long distance through the column was required. Therefore, this procedure is not reliable in homogeneity studies, since shape and width of the bands are strongly dependent on the distance traversed during the elution. Furthermore, in some cases elution causes tailing of zones which showed no adsorption on the medium during the electrophoretic migration. From these observations it is clear that components originally separated, the mobilities of which do not differ very much, may overlap each other as a result of the transport through the column. By cutting the starch in the starch block technique, this complication does not occur.

As a rule cellulose is a better medium than starch in column electrophoresis for low molecular weight substances. FLODIN AND KUPKE[67] consider the difference in electrophoretic behaviour of these substances in paper and starch as a disadvantage of starch column electrophoresis. But this unpredictable behaviour would not really be a disadvantage if the resolving power were greater.

The principal reason why starch column electrophoresis is not so popular as the block and gel techniques is that it is not so easily manipulated as either of the two other methods and does not present appreciable advantages.

## ACKNOWLEDGEMENT

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# CHROMATOGRAPHIC METHODS FOR THE IDENTIFICATION OF BIOGENIC AMINES*

JORGE AWAPARA, VIRGINIA E. DAVIS AND ODETTE GRAHAM
*The Rice Institute, Biology Department, Houston, Tex. (U.S.A.)*

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The term "Biogenen Amine" was first used by GUGGENHEIM in 1920, to describe a large group of compounds with basic properties, occurring naturally and possessing some properties in common. Many methods have been used for their isolation and identification. No systematic methods have been developed to identify them as a group. In animals and most bacteria, amines are usually the product of decarboxylation of amino acids and/or their derivatives. This limits the number of basic compounds that would occur naturally. Several methods have been reported for the separation of known amines by means of paper chromatography[1,2]. Direct chromatography of tissue extracts would be unsatisfactory since the concentration of amino acids in tissues is greater than the concentration of amines. This would mask any amines present in small amounts; also the amines would be indistinguishable from amino acids by direct development of color with ninhydrin. Weakly acidic cation exchange resins have been used in special cases. Amberlite IRC-50 has been used to separate adrenaline from nor-adrenaline[3-5] and also to separate some bases from urine[6]. It has been used in columns for the chromatography of basic amino acids[7]. In our laboratory we succeeded in separating basic amino acids and few amines using columns of buffered Amberlite IRC-50 and gradient elution. Buffers of increasing molarity were used, but the results were not good. Many amines failed to develop sharp boundaries and others decomposed in the presence of large amounts of salts. We abandoned this method in favor of partition chromatography on cellulose columns. Good separations were obtained in a few trial runs. In order to use cellulose as the stationary phase it was necessary to find a method to separate the amines from amino acids prior to chromatography and to obtain the solution of amines free of salts. We have succeeded in obtaining such solutions.

The methods reported here are methods for the quantitative separation of amines from amino acids and for the fractionation of amines by partition chromatography on cellulose columns and filter paper.

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## MATERIALS AND METHODS

### *Reference compounds*

Amino acids and amines used were all obtained from the California Corporation for Biochemical Research in the best grades available.

### *Resin*

Amberlite CG-50, Type 2, with a screen grading of approximately 200 (passing 200 mesh) was used. This resin was purified according to the procedure described by HIRS, MOORE AND STEIN for the purification of Amberlite IRC-50[8]. (This is the same type resin as CG-50 but of a different grade.) When the purification was completed, the resin was converted to the hydrogen form and stored.

### *Powder cellulose*

The powder cellulose was obtained from Brown Company, Boston, and is sold under the name of Solka-Floc. The cellulose was obtained with a screen grading of 200 mesh. Before use the powder cellulose was purified by washing several times with 1 *N* sodium hydroxide and 1 *N* hydrochloric acid. The cellulose was washed with distilled water and afterwards with alcohol. The cellulose was then dried by placing it under infrared lamps.

### *Detection of fractions*

The fractions obtained from each column were analyzed by determining their absorbancy at 279 m$\mu$ with a Beckman Model DU Spectrophotometer. An aliquot was removed from each fraction and analyzed by the ninhydrin method of MOORE AND STEIN[9]. Other aliquots were taken and analyzed by paper chromatography. The papers were treated with (a) ninhydrin and (b) diazotized sulfanilic acid.

## EXPERIMENTAL RESULTS

### 1. *Separation of mono- and dicarboxylic amino acids from amines and basic amino acids*

One of the difficulties in detecting small amounts of amines in tissues is that they are indistinguishable from amino acids by many of the known tests. Extraction of amines by solvents has disadvantages; one must operate at extremely alkaline pH and solvent extraction is seldom specific for one group of compounds. To obtain the amines from tissues free of amino acids, we tried several methods. WINTERS AND KUNIN[10] suggested a scheme for the separation of basic amino acids by means of Amberlite IRC-50 buffered to pH 4.7. We tried the same method to separate amines from amino acids. The separation was complete but the amine fraction was contaminated with salts eluted from the resin. Amberlite IRC-50, being a carboxylic type resin, reaches equilibrium only slowly when operated in the acid form. To obtain an amine fraction free of salts we had to use the resin in the acid form. In order to attain equilibrium the resin was allowed to remain in contact with the solution for one hour and with constant shaking. We finally decided on the following method. An amount of purified resin was added to the mixture of amino acids and amines. The amount used was in excess of the amount needed to reach saturation of the resin. After shaking the mixture in a mechanical shaker for one hour, the resin and solution were poured in a 1 × 15 cm column containing 1 g of the resin. After the resin had settled, the column was washed with water until all the amino acids were washed out of the column. The resin containing the amines and the basic amino acids was eluted with 2 × 10 ml of 4 *N* acetic acid. Recovery experiments were carried out using individual mono-carboxylic amino acids and a number of amines. The results are shown in Table I. It is clear from Table I that mono-carboxylic amino acids are not retained by the resin and that they can be recovered quantitatively in the effluent. It is also noted that the amines are retained quantitatively and eluted quantitatively from the resin bed. The analyses of both amines and amino acids were carried out by ultraviolet spectrophotometry and by the ninhydrin method. Verification of the degree of fractionation was possible by paper chromatography. By this method the separation is complete and quantitative. In Table II we show that a mixture of an amino acid and its corresponding amine can be separated quantitatively.

TABLE I

RECOVERY OF AMINO ACIDS FROM COLUMNS OF AMBERLITE CG-50 AND RETENTION OF AMINES

| *Compound added 5 μmoles* | *Found in effluent μmoles* | *Eluted from resin μmoles* | *% Recovery* |
|---|---|---|---|
| Glycine | 5.22 | — | 104.4 |
| Alanine | 5.18 | — | 105.6 |
| Threonine | 5.04 | — | 100.8 |
| Serine | 5.52 | — | 110.0 |
| γ-Aminobutyric acid | 5.02 | — | 100.4 |
| Leucine | 4.84 | — | 96.8 |
| Phenylalanine | 5.20 | — | 104.0 |
| Tyrosine | 5.02 | — | 100.4 |
| Tryptophan | 4.65 | — | 93.0 |
| Arginine | — | 5.21 | 104.2 |
| Lysine | — | 5.00 | 100.0 |
| Histidine | — | 5.00 | 100.0 |
| Phenylethylamine | — | 5.53 | 111.0 |
| Tryptamine | — | 5.03 | 100.6 |
| Tyramine | — | 4.94 | 98.8 |
| Serotonin | — | 5.02 | 100.4 |
| Epinephrine | — | 5.07 | 101.4 |
| Norepinephrine | — | 4.79 | 95.8 |
| Hordenine | — | 5.07 | 101.4 |
| 3-Hydroxytyramine | — | 4.97 | 99.4 |

## 2. *Column chromatography*

A number of preliminary experiments were carried out to find the best solvents for fractionating amines. Mixtures of several reference amines were chromatographed on

TABLE II

SEPARATION OF AMINO ACIDS AND AMINES WITH AMBERLITE CG-50

| *Added to resin 5 µmoles each* | *Recovered by ninhydrin method µmoles* | *%* | *by ultraviolet absorption µmoles* | *%* |
|---|---|---|---|---|
| Tyrosine | 4.66 | 93.2 | 5.04 | 100.8 |
| Tyramine | 4.71 | 94.2 | 5.19 | 103.8 |
| 5-Hydroxytryptophan | 5.44 | 108.8 | 5.31 | 106.2 |
| Serotonin | 4.82 | 96.4 | 5.05 | 101.0 |
| Tryptophan | 5.31 | 106.2 | 4.92 | 98.4 |
| Tryptamine | 5.28 | 106.6 | 5.17 | 103.4 |
| Phenylalanine | 5.47 | 109.4 | — | — |
| Phenylethylamine | 5.28 | 105.6 | — | — |

filter paper to determine their $R_F$ values in several solvents. Of the solvents tried butanol–acetic acid–water in a 4:1:1 proportion and 4:1:1.6 proportion gave very good results. Best results were obtained with 2-butanone–propionic acid–water in a 3:1:1.2 proportion. Unfortunately this solvent mixture cannot be used in columns although it gives excellent results on paper. In columns there are many disadvantages; the presence of the ketone interfered with the detection of compounds absorbing ultraviolet light. Too, the development of the ninhydrin color is inhibited. We used this solvent to verify the homogeneity of the fractions obtained from columns.

The procedure used for the chromatography of known mixtures of amines was the same every time. The columns were packed by several additions of a suspension of cellulose powder in solvent. Occasionally slight pressure was applied. The solvent to be used was allowed to wash the column until the effluent gave a low and constant reading when read at 279 mµ and no color when tested with the ninhydrin reagent. When the column was ready the mixture of amines was pipetted, usually in a 1 ml volume, made up with the chromatographic solvent. The solvent was then allowed to flow at a rate of about 4 ml per hour. Fractions of 1 ml volume were collected. Each fraction was analyzed as follows: (a) absorption at 279 mµ, (b) color produced with ninhydrin, and (c) chromatography on filter paper with a solvent different from that used on the column.

(*A*) *Fractionation with butanol–acetic acid–water, 4:1:1.* This solvent gave good separation of several amines on filter paper. A mixture of 2 µmoles of each of the following amines was prepared: valamine, phenylethylamine, tryptamine, tyramine, serotonin, 3-hydroxy-tyramine, epinephrine, norepinephrine, ethanolamine, histamine, cadaverine, putrescine, arginine, lysine, threamine. The mixture was chromatographed on a column as previously described. The results obtained are shown in Fig. 1.

(*B*) *Fractionation with butanol–acetic acid–water, 4:1:1.6.* The results obtained with the mixture previously described are satisfactory for some of the amines. The aromatic compounds separate well but the more polar substances tend to be retarded and as a result of this retardation the boundaries become distorted. We thought that by increasing the polarity of the solvent slightly we would increase the rate of migration of the more polar substances without affecting materially the less polar substances. For this we chose the solvent mixture of butanol–acetic acid–water in a proportion of 4:1:1.6. The results obtained with this solvent using the same

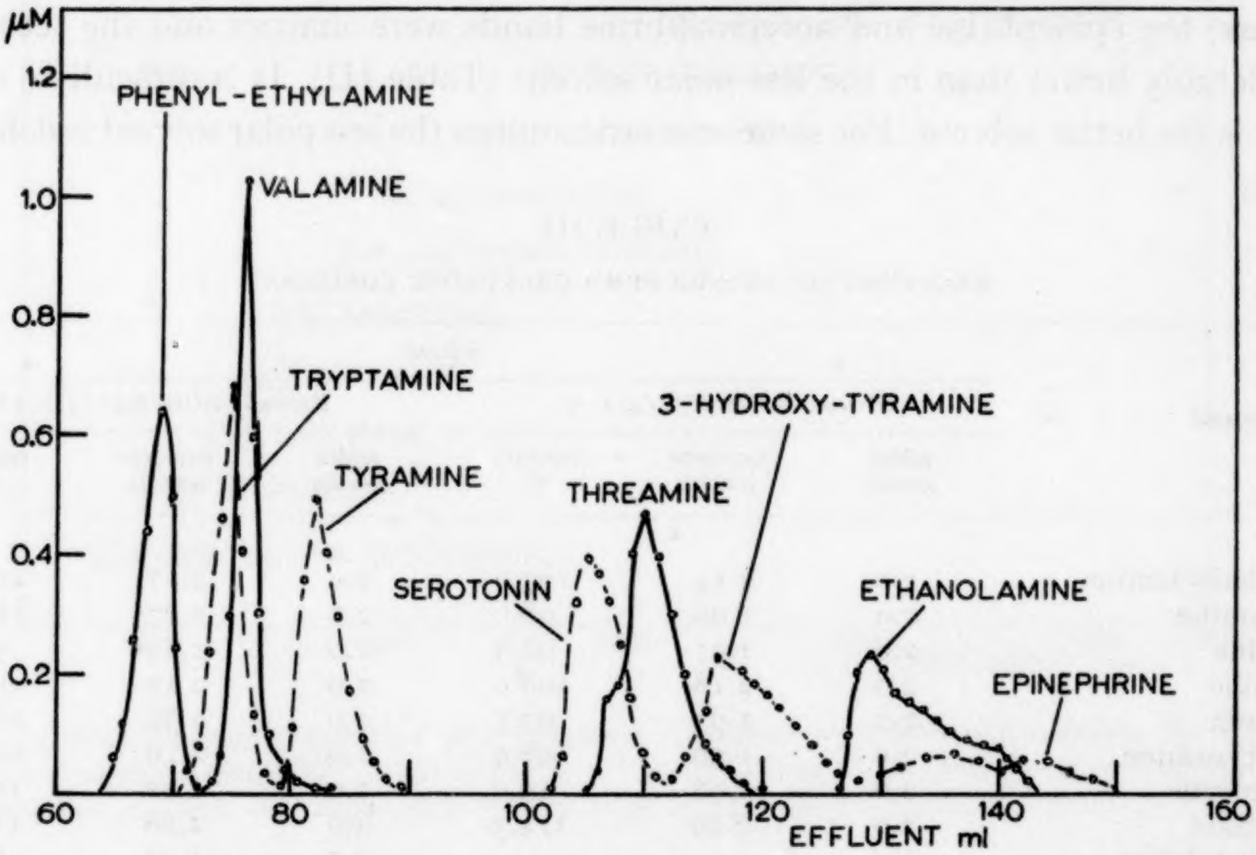

Fig. 1. Chromatographic separation of a mixture of amines on a cellulose column (80 × 1 cm). Solvent: butanol–acetic acid–water, 4:1:1. Rate of flow: 4 ml per hour. Solid line represents concentration of effluent measured by the ninhydrin method. Broken lines represent concentration measured by absorption at 279 m$\mu$.

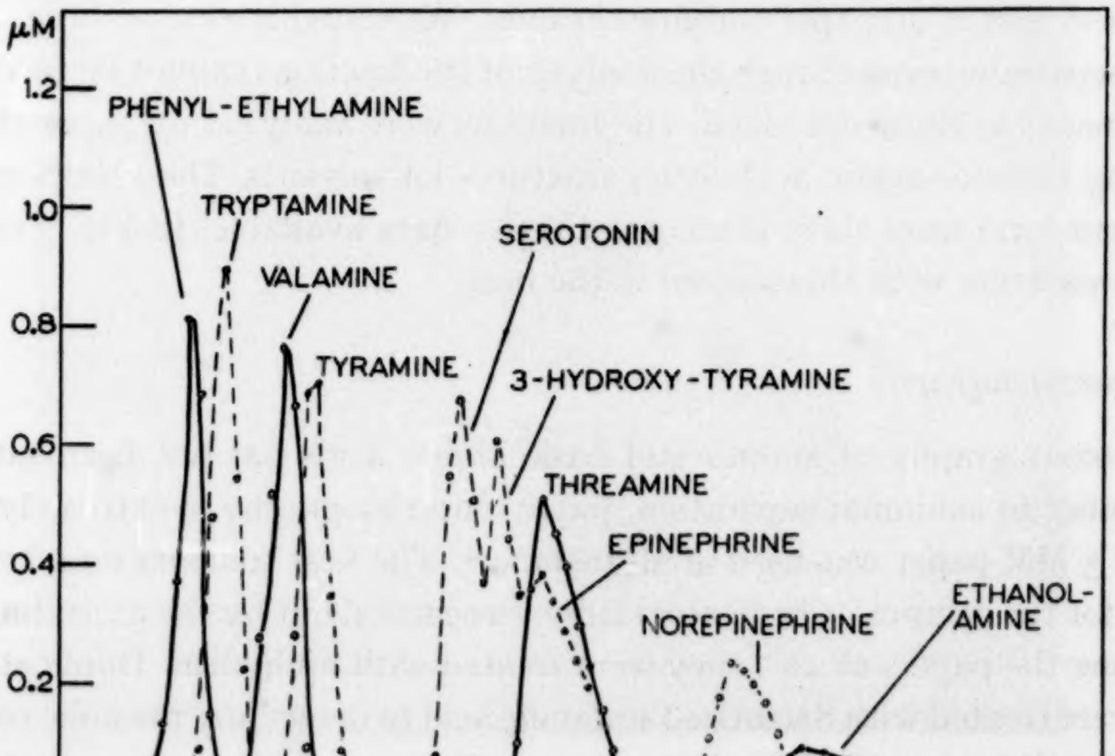

Fig. 2. Chromatographic separation of a mixture of amines in a cellulose column (80 × 1 cm). Solvent: butanol–acetic acid–water, 4:1:1.6. Conditions were the same as those described in Fig. 1.

compounds as those used in the previous column are shown in Fig. 2. The higher polarity of this solvent over the previous one causes most compounds to move faster. In the less polar solvent several amines and the basic amino acids failed to yield clear-cut boundaries. No separation of them was accomplished. With the more polar mixture, the epinephrine and norepinephrine bands were sharper and the recoveries considerably better than in the less polar solvent (Table III). It is difficult to decide which is the better solvent. For some aromatic amines the less polar solvent is definitely better.

TABLE III

RECOVERY OF AMINES FROM CELLULOSE COLUMNS

| *Compound* | *Solvent* | | | | | |
|---|---|---|---|---|---|---|
| | *Butanol–AcOH–$H_2O$ (4:1:1)* | | | *Butanol–AcOH–$H_2O$ (4:1:1.6)* | | |
| | *added µmoles* | *recovered µmoles* | *recovery %* | *added µmoles* | *recovered µmoles* | *recovery %* |
| Phenylethylamine | 2.0 | 2.14 | 107.0 | 2.0 | 2.17 | 108.5 |
| Tryptamine | 2.0 | 1.96 | 98.0 | 2.0 | 2.07 | 103.5 |
| Valamine | 2.0 | 1.97 | 98.5 | 2.0 | 1.85 | 92.5 |
| Tyramine | 2.0 | 2.18 | 109.0 | 2.0 | 2.12 | 106.0 |
| Serotonin | 2.0 | 1.95 | 97.5 | 2.0 | 2.30 | 115.0 |
| 3-OH-tyramine | 2.0 | 1.96 | 98.0 | 2.0 | 2.10 | 105.0 |
| Epinephrine | 2.0 | 0.98 | 49.0 | 2.0 | 2.01 | 100.5 |
| Threamine | 2.0 | 2.29 | 114.5 | 2.0 | 2.28 | 114.0 |
| Norepinephrine | 2.0 | | | 2.0 | 1.22 | 61.0 |
| Ethanolamine | 2.0 | 1.98 | 99.0 | 2.0 | 0.86 | 43.0 |

(*C*) *Fractionation with 2-butanone–propionic acid–water, 3:1:1.2.* This solvent gave the best results on paper chromatography. We tested it as a possible solvent for columnar separation even though the analysis of the fractions cannot be carried out by the same means as those described. The fractions were analyzed by paper chromatography using butanol–acetic acid–water mixtures for solvents. The results are plotted in a different form since there is no quantitative data available. In Fig. 3 one can see that the separation with this solvent is the best.

### 3. *Paper chromatography*

Paper chromatography of amines and basic amino acids has not been satisfactory. As an adjunct to columnar separation, paper chromatography is extremely valuable. Whatman 3 MM paper was used in all instances. The best solvents found were those described for the columns. Chromatography was carried out by the ascending method. After drying the papers at 40°, they were treated with ninhydrin. Duplicate chromatograms were treated with diazotized sulfanilic acid to detect any phenolic compounds. In Fig. 4 are shown some selected separations. The separation is not only satisfactory but the spots obtained are compact and well defined. Of the solvents used 2-butanone-propionic acid–water was the best. We have tried a number of solvents containing ammonia but the results were not good. In many cases there was poor definition of

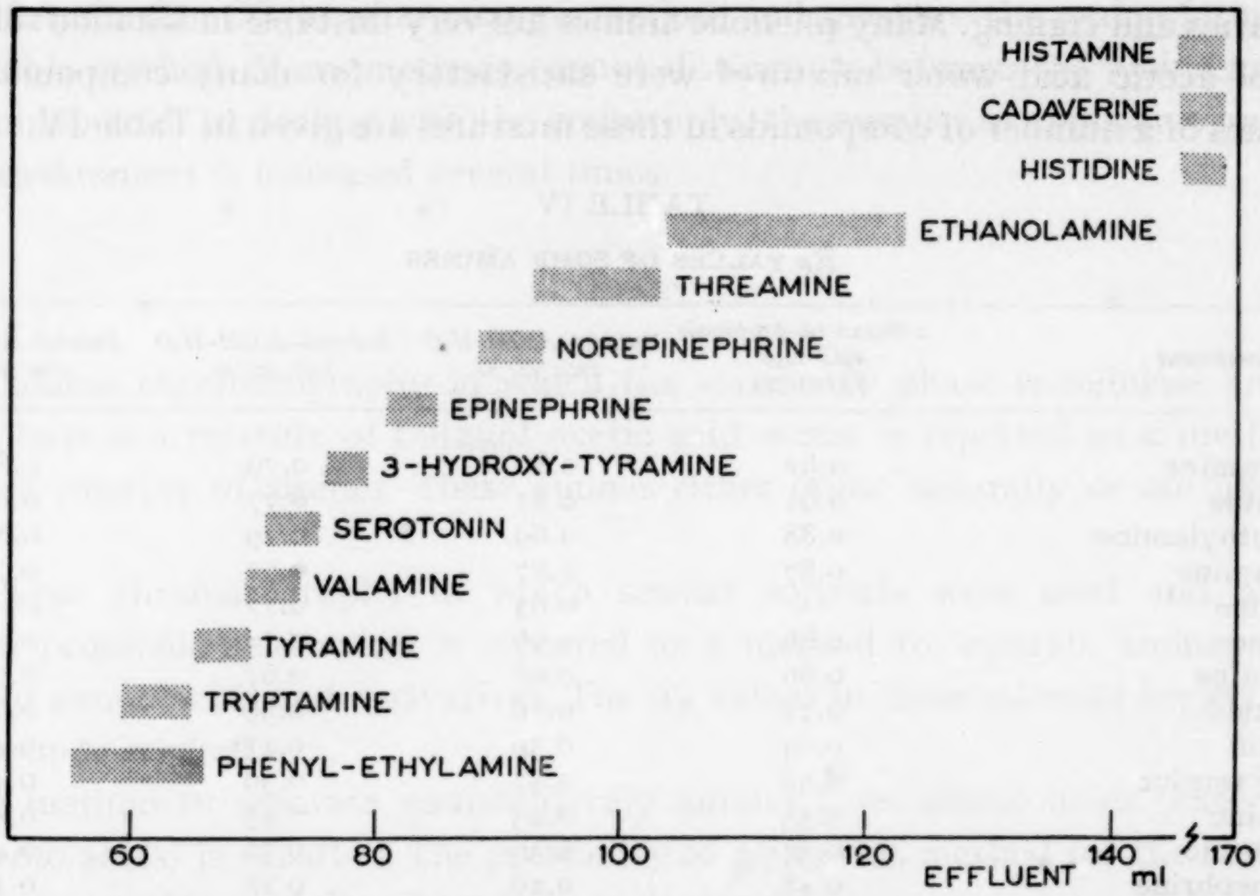

Fig. 3. Chromatographic separation of a mixture of amines on a cellulose column (80 × 1 cm). Solvent: 2-butanone–propionic acid–water, 3:1:1.2. Analyses of fractions carried out by paper chromatography. Each bar represents the volume of effluent in which the fraction was found.

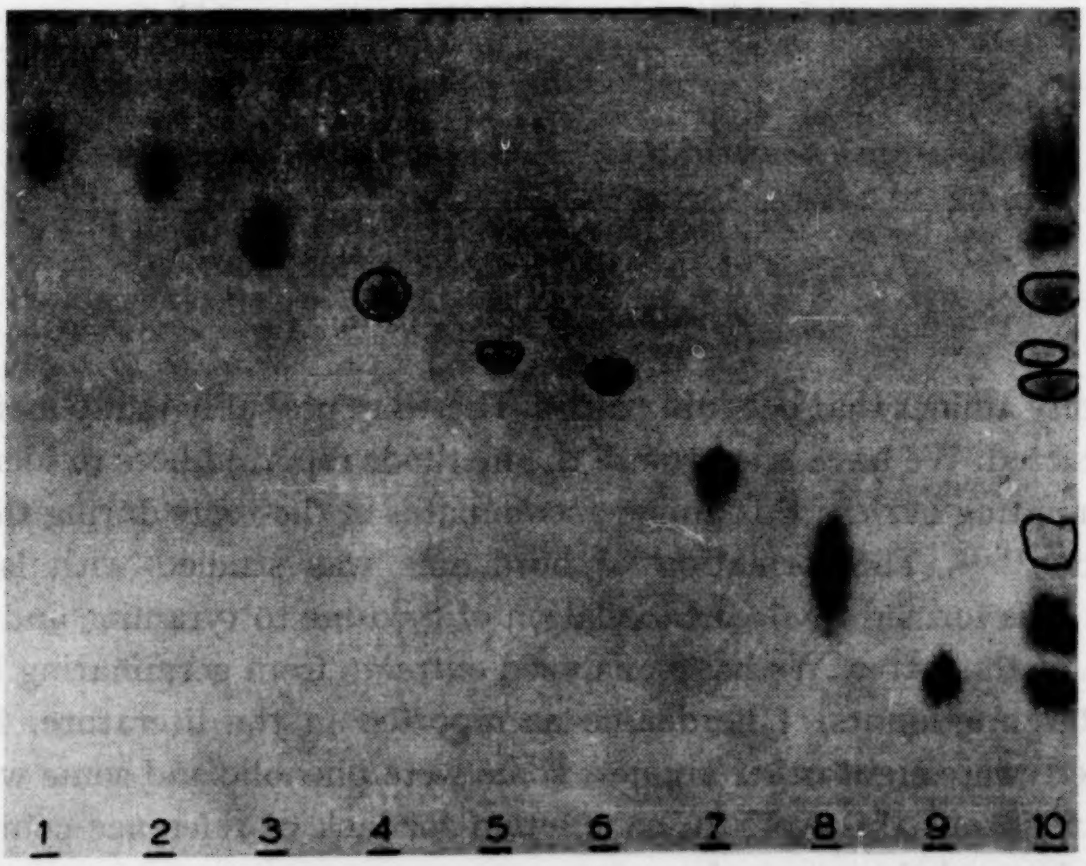

Fig. 4. Chromatographic separation of a mixture of amines on filter paper (Whatman 3 MM). Solvent: 2-butanone–propionic acid–water, 3:1:1.2. Spots marked with pencil give colors that do not show well in photographs. (1) Phenylethyl amine, (2) Tryptamine, (3) Tyramine, (4) Serotonin, (5) Epinephrine, (6) Norepinephrine, (7) Histamine, (8) Putrescine, (9) Lysine, and (10) mixture of all of them.

boundaries and trailing. Many phenolic amines are very unstable in alkaline solutions. Butanol–acetic acid–water mixtures were satisfactory for many compounds. The $R_F$ values of a number of compounds in these mixtures are given in Table IV.

TABLE IV

$R_F$ VALUES OF SOME AMINES

| *Compound* | *2-Butanone–propionic acid–$H_2O$ 75:25:30* | *Butanol–AcOH–$H_2O$ 120:30:30* | *Butanol–AcOH–$H_2O$ 120:30:40* | *Butanol–AcOH–$H_2O$ 120:30:50* |
|---|---|---|---|---|
| Norleucamine | 0.92 | 0.80 | 0.79 | 0.80 |
| Leucamine | 0.91 | 0.81 | 0.77 | 0.81 |
| Phenylethylamine | 0.88 | 0.69 | 0.79 | 0.81 |
| Norvalamine | 0.87 | 0.77 | 0.71 | 0.76 |
| Hordenine | — | 0.63 | 0.77 | — |
| Valamine | 0.86 | 0.66 | 0.74 | 0.75 |
| Tryptamine | 0.86 | 0.60 | 0.67 | 0.73 |
| Tyramine | 0.77 | 0.58 | 0.59 | 0.67 |
| Serotonin | 0.64 | 0.38 | 0.41 | 0.47 |
| 3-OH-tyramine | 0.58 | 0.41 | 0.39 | 0.46 |
| Threamine | 0.55 | 0.43 | 0.43 | 0.47 |
| Epinephrine | 0.54 | 0.30 | 0.32 | 0.45 |
| Norepinephrine | 0.48 | 0.26 | 0.28 | 0.40 |
| Seramine | 0.46 | 0.34 | 0.34 | 0.38 |
| Agmatine | 0.46 | 0.21 | 0.20 | 0.34 |
| Histamine | 0.43 | 0.20 | 0.20 | 0.30 |
| Cadaverine | 0.39 | 0.16 | 0.14 | 0.26 |
| Putrescine | 0.32 | 0.13 | 0.11 | 0.20 |
| Arginine | 0.26 | 0.09 | 0.04 | 0.17 |
| Glutamine | 0.25 | 0.11 | 0.16 | 0.25 |
| Lysine | 0.24 | 0.06 | 0.07 | 0.14 |
| Histidine | 0.23 | 0.09 | 0.08 | 0.16 |
| Carnosine | 0.23 | 0.08 | 0.08 | 0.15 |
| Methyl-histidine | 0.23 | 0.08 | 0.10 | 0.18 |
| Thiohistidine | 0.14 | 0.11 | 0.08 | 0.18 |
| Spermine | 0.05 | — | — | — |

## POSSIBLE APPLICATIONS

There are many amines that occur in nature. There are probably many more that have not been detected. We have tried to use the methods reported here to identify hordenine in germinating barley; this amine accumulates in the roots during the first week of germination[11, 12]. The formation of hordenine was studied with labeled compounds[13–15]. It is formed by decarboxylation of tyrosine to tyramine and subsequent methylation of the latter. We have analyzed extracts from germinating barley roots and found large amounts of hordenine as reported in the literature. In addition, however, there were eight other amines; some were phenolic and some were not. We have not carried out this work to completion for lack of reference compounds and because it is beyond the scope of our interest.

As a possible extension of this work, we believe that one can develop a relatively easy method for measuring amino acid decarboxylases. The separation of an amine and its parent amino acid is complete. Thus, one can remove the excess substrate by means of Amberlite CG-50 after enzymic action, and measure the amine formed by any suitable method. Many methods cannot distinguish between the amine and the parent amino acid. In dealing with the amine only, the number of applicable methods to its measurement is increased several times.

## SUMMARY

1. Column chromatography in which the stationary phase is cellulose and the mobile phase is a mixture of butanol–acetic acid–water is reported as a method to separate a number of amines. These amines either occur naturally or are likely to occur.

2. Paper chromatography in which similar solvents were used and also 2-butanone–propionic acid–water is reported as a method to separate amines and a number of amino acids and derivatives. The $R_F$ values in these solvents are given for 26 compounds studied.

3. A method to separate quantitatively amines from amino acids (except the basic amino acids) is reported. The possibility of using this method for the measurement of some amino acid decarboxylases is discussed.

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# RADIOAUTOGRAPHIC DETECTION OF METABOLITES OF $^{35}$S-DL-CYSTINE

D. CAVALLINI, C. DE MARCO, B. MONDOVÌ
*Institutes of Biological Chemistry of the Universities of Modena and Rome*
AND
L. TENTORI
*Laboratory of Biology of the Istituto Superiore di Sanità, Rome (Italy)*

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As part of a general program of investigation of the metabolism of cystine in mammals, this paper presents the results of experiments made using labeled cystine in the rat. ARNSTEIN AND CRAWHALL[1] and AWAPARA[2,3] were the first to use labeled cystine for this type of investigation. In the present paper we describe the occurrence of a number of unsuspected cystine metabolites as detected by paper radioautography of the kidney extract and the urine of the rat injected with labeled cystine. A preliminary identification of some of the detected compounds has been possible by using the criterion of identity based on the fingerprint-like comparison between the radioactive spot of the unknown and the ninhydrin spot of the authentic sample of the suspected product added on the same chromatogram.

## METHODS AND MATERIALS

$^{35}$S-DL-cystine was obtained from Amersham, England. It had an activity of 1 mC/6.9 mg. 2 mC were dissolved in 0.3 ml 1 *N* HCl, diluted to 2 ml with water, divided into two portions and injected intravenously into two male rats weighing 200 g. One rat was killed after 2 h, the kidneys were removed and an extract suitable for chromatography was obtained as described by AWAPARA[4]. The final aqueous extract (2 ml) was stored in the deep freeze. The other rat was kept in a metabolic cage and the urine of 24 h was collected under toluene. The sample was diluted to 30 ml with the washings of the funnel and was stored in the deep freeze.

Chromatograms were made on Whatman No. 4 paper using water-saturated phenol in the first direction and a mixture of collidine and lutidine saturated with one volume of water, in the second direction. Radioautograms were made by lightly pressing the dried chromatograms on a 30 × 40 cm X-ray film in the dark for a suitable length of time (10–20 days). After radioautography, the same chromatograms were developed with ninhydrin in order to locate amino acids and other ninhydrin-reacting compounds.

## RESULTS

The radioautogram of 0.1 ml of kidney extract from the rat injected with labeled cystine is reproduced in Fig. 1. A very large number of compounds appears on this autogram, at least 12 different radioactive spots have been enumerated. Among these only taurine and hypotaurine may be easily identified by their unequivocal chromatographic location. A few other spots have been identified after careful control with an authentic sample of the suspected compound. All the remaining products are unknown.

Fig. 2 shows the same chromatogram used for the radioautography of Fig. 1, after the reaction with ninhydrin. It can be seen that, with the exception of taurine, all the radioactive compounds are present in such low amount that they cannot be detected with ninhydrin, although a number of them certainly have a ninhydrin-reactive $NH_2$-group. In order to emphasize this point more strongly, the position of some of the radioactive spots seen in Fig. 1 have been marked by pen on the chromatogram of Fig. 2.

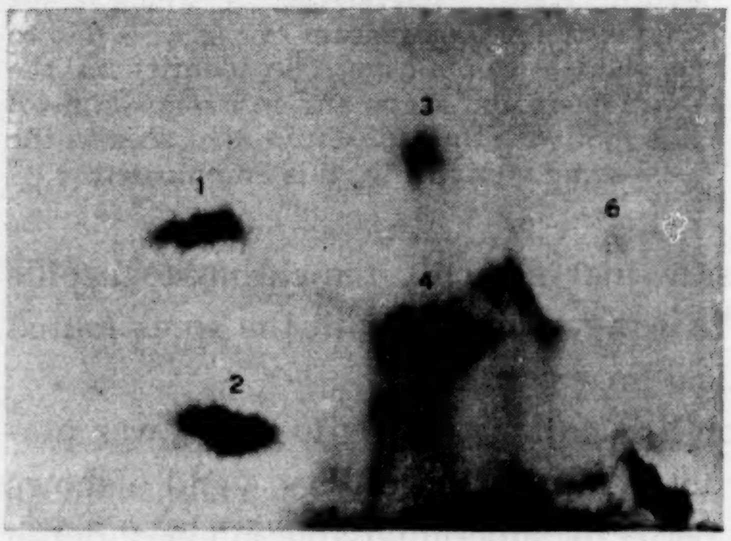

Fig. 1. Radioautogram (20 days) of 0.1 ml of kidney extract from a rat injected with 1 mC of $^{35}$S-DL-cystine. Spots: 2 = hypotaurine; 4 = taurine. For the identification of the remaining spots see text. Sample applied at the right lower corner.

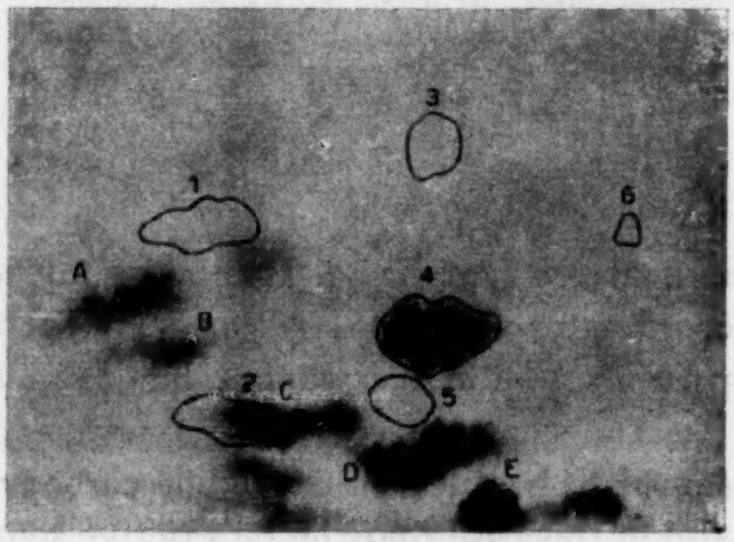

Fig. 2. The same chromatogram used for the radioautography of Fig. 1, after development with ninhydrin. The outlines of the more important radioactive spots seen in Fig. 1 have been marked in this chromatogram. Some of the ninhydrin-reacting spots have been identified as follows: A = leucines; B = valine; C = alanine; D = glycine; E = glutamic acid.

Very recently thiotaurine has been detected in the urine of rats fed with unlabeled cystine[5]. We deemed it of interest to confirm this finding using the labeled compound. The radioactive spot marked as No. 3 in Fig. 1 shows a chromatographic behavior like that expected for thiotaurine. In order to establish the identity of this spot with thiotaurine, a chromatogram was made with 0.2 ml of the kidney extract to which 50 $\mu$g of a pure sample of synthetic thiotaurine[6] had been added. After contact with the X-ray film for 10 days, the chromatogram was removed and developed with ninhydrin. Fig. 3 represents the radioautography of the same chromatogram developed with ninhydrin which is shown in Fig. 4. It can be seen that in the chromatogram the new spot of the added thiotaurine is now present, located in the same area as the

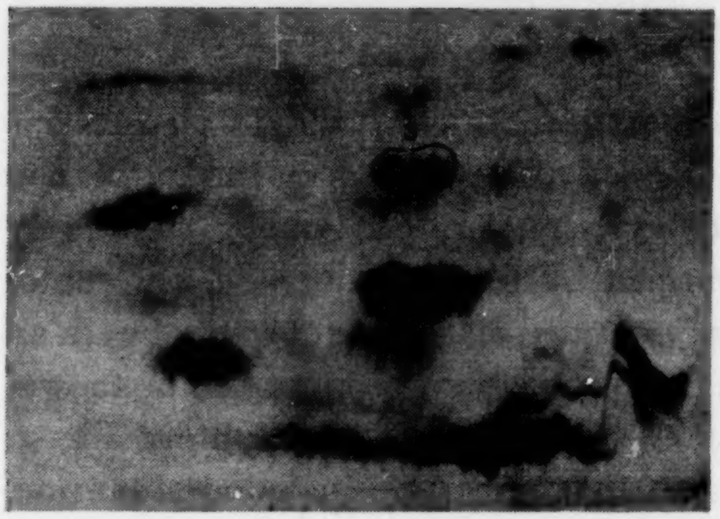

Fig. 3. Radioautogram (10 days) of 0.2 ml kidney extract from a rat injected with 1 mC $^{35}$S-DL-cystine, to which 50 $\mu$g of unlabeled synthetic thiotaurine had been added.

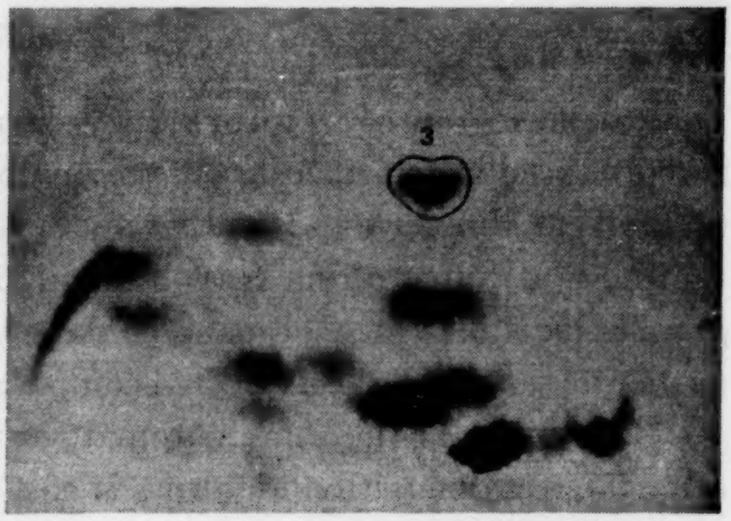

Fig. 4. The same chromatogram used for the radioautogram of Fig. 3, developed with ninhydrin. The spot marked No. 3, which is not seen in the chromatogram of Fig. 2, is due to the added thiotaurine. The identity in the position, in the shape and in every detail of this spot with the radioactive spot seen in the same area in Fig. 3, is emphasized.

radioactive spot No. 3. It may be further seen that not only the chromatographic coordinates of the two spots are identical but that the outlines of both the spots match perfectly well in every detail, like two identical fingerprints.

The same procedure was applied to spot No. 1 which, by its location, was presumed to be due to thiazolidine carboxylic acid. Comparison of Figs. 5 and 6 shows also in this case that the radioactive spot is perfectly superimposable in every detail on the ninhydrin spot of the same chromatogram made with added pure thiazolidine carboxylic acid.

The identification of spots No. 3 and No. 1 respectively as thiotaurine and thiazolidine carboxylic acid was further confirmed by their oxidation to taurine and

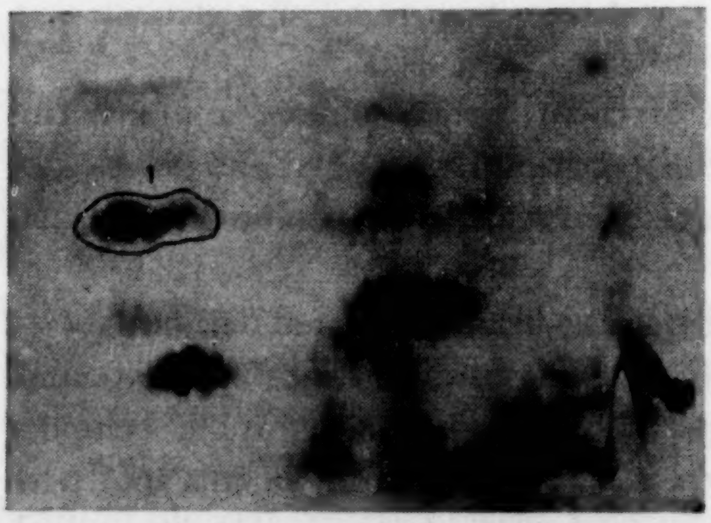

Fig. 5. Radioautogram (10 days) of 0.2 ml kidney extract from a rat injected with 1 mC $^{35}$S-DL-cystine, to which 50 $\mu$g of unlabeled synthetic thiazolidine carboxylic acid had been added.

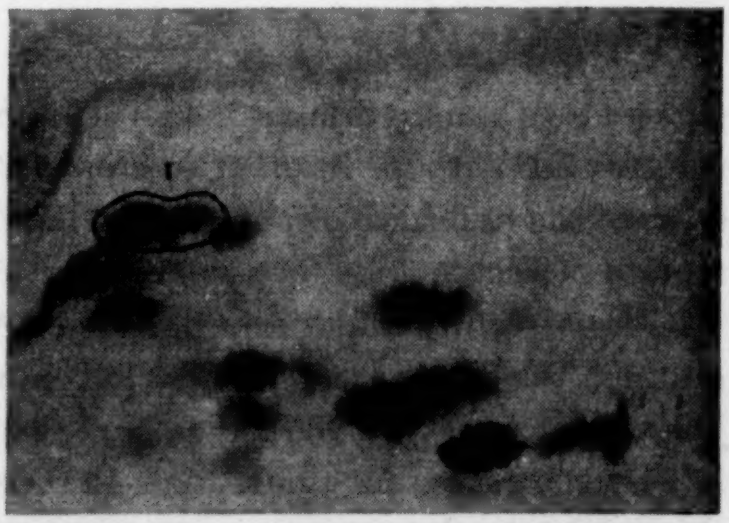

Fig. 6. The same chromatogram used for the radioautogram of Fig. 5, developed with ninhydrin. The spot marked No. 1 which is not seen in the chromatogram of Figs. 2 and 4 is due to the added thiazolidine carboxylic acid. The identity in the position, in the shape and in all details of this spot with the radioactive spot seen in the same area in Fig. 5, is emphasized.

to cysteic acid. Using a radioautogram as a guide, the areas of the two spots were cut out of the respective chromatograms. The fragments were eluted with water, the solution was treated with 1 ml 30% hydrogen peroxide in the presence of a trace of molybdate[7], and two new chromatograms and radioautograms were made with the final liquids. Among minor products, a spot of taurine and one of cysteic acid were detected as required for thiotaurine and thiazolidine carboxylic acid respectively.

Radioautography and chromatography of a 0.2 ml sample of the urine of the second rat are shown in Figs. 7 and 8. The chromatographed sample has been added with 50 μg of a pure sample of cysteinsulfonate[8] in order to show, by the same criterion of identification used above, the identity of spot No. 6 with cysteinesulfonate. The chromatogram of the urine without the addition of cysteinesulfonate obviously lacks spot No. 6.

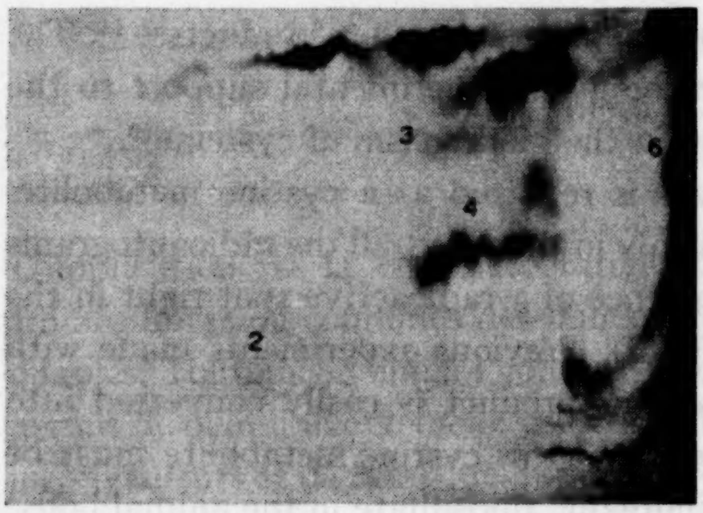

Fig. 7. Radioautogram (14 days) of 0.2 ml urine of rat injected with 1 mC $^{35}$S-DL-cystine, added with 50 μg of synthetic unlabeled cysteinesulfonate. Spots: 2 = hypotaurine; 3 = thiotaurine; 4 = taurine; 6 = cysteinesulfonate.

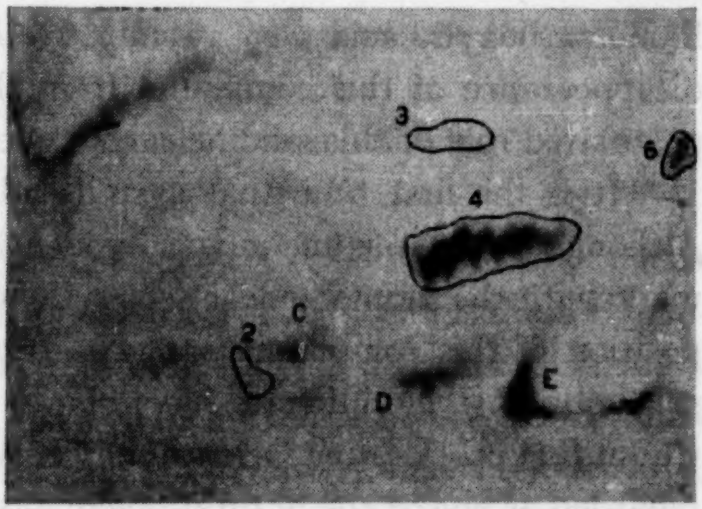

Fig. 8. The same chromatogram used for the radioautography of Fig. 7, developed with ninhydrin. The outline of some radioactive spots seen in Fig. 7 have been marked in this chromatogram. Some of the ninhydrin-reacting spots have been identified as follows: C = alanine; D = glycine; E = glutamic acid.

The radioautogram of the urine is different from that of the kidney extract. Three very strong unidentified new spots appear which are not seen in the kidney extract. Moreover the spots identified in the kidney extract, namely, taurine, hypotaurine, thiotaurine, and thiazolidine carboxylic acid, are much weaker in the urine, probably owing to their higher dilution. Cysteinesulfonate has been detected in the urine; it is likely that the small spot falling in the same area in the autograms of kidney extracts (spots No. 6 of Figs. 1 and 2) is cysteinesulfonate itself.

## DISCUSSION

Some of the metabolites of DL-cystine detected on the radioautograms have been identified. Among them are those present in larger amount. It is possible that some of the spots may be due to an artifact or may have originated from the unnatural portion of the racemic cysteine injected. Investigation is being continued in order to elucidate these points. Apart from this limitation, the large number of the metabolites detected indicates the occurrence in the animal body of a number of metabolic pathways of DL-cystine yet to be discovered.

The identification of thiotaurine among the detected metabolites is of particular interest. It provides a confirmation of a previous finding and at the same time it serves to exclude the possibility that the thiotaurine first identified in the urine of cystine-fed rats[5] had a bacterial origin. Indeed, in the above experiments, cystine had been injected in the veins and thiotaurine was detected in the kidney extract, thus making any interference of the intestinal bacteria very unlikely.

The occurrence of thiazolidine carboxylic acid is another point which deserves attention. This product arises spontaneously by the interaction of cysteine with formaldehyde[9]. Enzymic systems have recently been described which oxidize thiazolidine carboxylic acid very rapidly to diformylcystine and other products[10, 11]. The disappearance of this compound from the urine gives experimental support to the postulated role of thiazolidine carboxylic acid in the metabolism of cysteine[10].

It is the first time that cysteinesulfonate is reported as a cystine metabolite. Although it has been unequivocally identified only in the urine, all the radioautograms made with the kidney extract show the occurrence of a radioactive spot right in the position of this compound (spot No. 6 of Fig. 1). Previous experiments made with synthetic cysteinesulfonate have shown that this product is easily converted into thiosulfate by the rat[12]; accordingly it is likely that this cystine metabolite must be regarded as one of the natural precursors of inorganic thiosulfate in the animal body.

This work forms part of a research program on the biochemistry of sulfur, sponsored by the Comitato Nazionale Ricerche Nucleari.

## SUMMARY

Metabolites of $^{35}$S-DL-cystine have been detected in the kidney extract and in the urine of rats by means of radioautography. Comparison of the chromatograms containing added pure samples of the suspected radioactive spots with their radioautograms permitted the identification of some of the detected compounds. The following cystine metabolites have been identified: taurine, hypotaurine, thiotaurine, thiazolidine carboxylic acid, cysteinesulfonate.

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# SÉPARATION DES ACIDES AMINÉS DES MILIEUX BIOLOGIQUES COMPLEXES

## V. DESCRIPTION D'UN COUPLAGE DE MÉTHODES CHROMATOGRAPHIQUES ET ÉLECTROPHORÉTIQUES*

GÉRARD BISERTE, THÉRÈSE PLAQUET-SCHOONAËRT,
PAUL BOULANGER ET PIERRE PAYSANT
*Laboratoire de Chimie Biologique, Faculté de Médecine et de Pharmacie, Lille (France)*

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L'application de la chromatographie de partage sur papier à l'étude des acides aminés libres ou combinés des milieux biologiques a déjà fourni des résultats remarquables. Toutefois, l'emploi continu de cette méthode depuis plus de dix ans nous a permis de définir exactement ses possibilités, mais en même temps de préciser certains inconvénients et de faire quelques réserves: l'apparition fréquente, dans la littérature, de nouveaux systèmes-solvants montre d'ailleurs bien que la satisfaction n'est pas totale!

### A. REVUE CRITIQUE DES POSSIBILITÉS SÉPARATIVES DE LA CHROMATOGRAPHIE BIDIMENSIONNELLE

### 1. *Couplage butanol–acide acétique et phénol aqueux*

Le type de chromatographie bidimensionnelle le plus employé est réalisé en première dimension avec le solvant butanol–acide acétique–eau (4:1:5) (PARTRIDGE[36]) et en seconde dimension avec le phénol saturé d'eau en atmosphère ammoniacale (CONSDEN *et al.*[7]) ou le phénol tamponné à pH 9.3 (LEVY ET CHUNG[27]). Avec ces solvants, la séparation des acides aminés d'un hydrolysat total de protéine est satisfaisante à condition que l'acide chlorhydrique soit complètement éliminé (voir la carte de PARRY[35]). Toutefois, la séparation de la sérine et du glycocolle, ainsi que celle de la leucine et de la phénylalanine ne sont pas complètes, et ceci constitue un inconvénient majeur pour le dosage chromatographique des acides aminés. D'autre part, la valine et la méthionine occupent exactement la même position, et l'artifice qui consiste à transformer la méthionine en méthionine-sulfone et (ou) en méthionine-sulfoxyde (DENT[8]) n'est pas toujours d'une application commode, car il y a très souvent dans les solutions étudiées une faible proportion de méthionine par rapport à la valine et l'identification est donc malaisée. De même, la caractérisation de la cystine n'est pas

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[*] Pour les mémoires antérieurs, voir références[3,4,6].

toujours facile: il est indispensable d'effectuer un second chromatogramme après oxydation* et de repérer alors l'acide cystéique.

L'interprétation des chromatogrammes bidimensionnels des fractions amino-acides des milieux biologiques (tissus animaux et végétaux, sérum, urine, etc.) est encore plus délicate et pose des problèmes particuliers très difficiles à résoudre (Fig. 1).

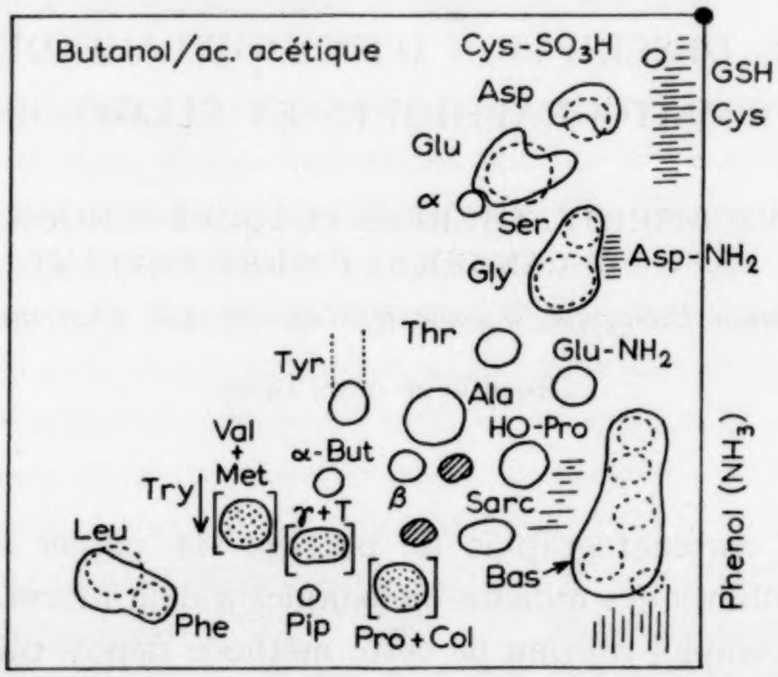

Fig. 1. Schématisation des principales difficultés rencontrées au cours de la chromatographie bidimensionnelle des acides aminés des milieux complexes. (Chromatographie dans le solvant butanol–acide acétique–eau (4 : 1 : 5) en première dimension et phénol saturé d'eau en atmosphère ammoniacale en seconde dimension.) Les cercles pointillés figurent l'emplacement théorique des acides aminés. Les surfaces entourées d'un trait plein correspondent aux positions habituellement observées au cours de l'étude d'un milieu biologique complexe. Les zones hachurées indiquent l'emplacement des plages "ninhydrine positive" d'interprétation délicate. Les surfaces ponctuées et entourées de parenthèses représentent des régions de chevauchement d'acides aminés. Dans la région de la proline (bien que la coloration jaune de cet aminoacide avec le réactif à la ninhydrine soit assez caractéristique) il est souvent difficile de conclure à la présence de la colamine. Les deux surfaces hachurées indiquent la position de la $\beta$-alanine et de l'acide $\gamma$-aminobutyrique dans un hydrolysat total de milieu biologique (l'acide chlorhydrique ayant été éliminé simplement par évaporation sous vide). Les abréviations suivantes ont été utilisées: Cys–$SO_3H$ = acide cystéique; Asp = acide aspartique; Glu = acide glutamique; $\alpha$ = acide $\alpha$-aminoadipique; Ser = sérine; Gly = glycocolle; Asp-$NH_2$ = asparagine; Glu-$NH_2$ = glutamine; Thr = thréonine; Tyr = tyrosine (lorsque la tyrosine se trouve à une concentration élevée, elle a tendance à traîner); Ala = alanine; $\beta$ = $\beta$-alanine; HO-Pro = hydroxyproline; Sarc = sarcosine; Bas = acides aminés basiques (ornithine, histidine, lysine, méthylhistidine) + ansérine + carnosine; Pro = proline; Col = colamine; Pip = acide pipécolique; Phe = phénylalanine; Leu = leucines; Try = tryptophanne; Val = valine; Met = méthionine; $\gamma$ = acide $\gamma$-aminobutyrique; T = acide $\beta$-aminoisobutyrique ou T-spot; $\alpha$-But = acide $\alpha$-aminobutyrique.

En effet, malgré une déminéralisation soignée, la région du chromatogramme occupée par les acides diaminés est généralement confuse. Elle se présente très souvent sous la forme d'une traînée dans laquelle la séparation de l'arginine, de la lysine et de l'histidine est imprécise. Cette observation est particulièrement nette dans le cas des urines qui contiennent une très forte proportion d'histidine et dans lesquelles peut se trouver un taux élevé de méthylhistidine, ainsi que de l'ansérine et de la carnosine, qui viennent se placer dans la même zone.

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[*] L'oxydation performique de l'hydrolysat total (SCHRAM *et al.*[43]) est préférable à l'oxydation par l'eau oxygénée à 110 volumes des substances déposées à la tache de départ.

Sur les chromatogrammes, certaines amines biologiques forment au-dessous de la zone des acides diaminés une tache confuse qu'il est difficile de décomposer exactement. On peut trouver dans ce groupe la $\beta$-mercaptoéthylamine.

De faibles quantités d'asparagine ne peuvent être décelées facilement sur les chromatogrammes bidimensionnels, car cette substance donne avec la ninhydrine une tache de coloration brunâtre qui vient se juxtaposer au groupe sérine–glycocolle. La taurine se place également au niveau de cette zone. Si les milieux biologiques n'ont pas été fractionnés sur une résine à échange de cations fortement acide (Dowex 50 ou Amberlite IR 120), qui retient tous les acides aminés mais ne fixe pas la taurine (BOULANGER, BISERTE ET COURTOT[6]), celle-ci ne peut être aisément identifiée.

Il est aussi difficile de distinguer nettement la cystine et le glutathion. Les différences de $R_F$ sont minimes et le glutathion à tendance à traîner. Pour identifier la glutathion, il est indispensable d'éluer très exactement la région correspondante (sans déborder sur la tache de l'acide glutamique et du glycocolle), d'hydrolyser totalement l'éluat et de chromatographier l'hydrolysat: la présence de cystine, d'acide glutamique et de glycocolle permet de conclure à la présence de glutathion.

D'autre part, l'acide $\gamma$-aminobutyrique se place très près de l'acide $\beta$-aminoisobutyrique (ou T-spot) et la séparation est peu satisfaisante.

La distinction entre l'acide $\delta$-aminovalérique et la valine est également délicate. Elle est possible avec des solutions témoins équimoléculaires, mais, dans le cas des milieux biologiques, où il y a souvent une disproportion très marquée entre les taux des deux substances, l'identification est souvent impossible.

La plupart des inconvénients que nous venons de signaler se retrouvent dans le couplage butanol–acide acétique et *m*-crésol–phénol tamponné à pH 9.3 (LEVY ET CHUNG[27]).

## 2. *Autres couplages bidimensionnels*

(*a*) *Système collidine–phénol.* Des difficultés comparables aux précédentes s'observent également avec le système collidine–phénol (voir DENT[8]) *. De plus, la variabilité de composition de la collidine, la sensibilité de ce système-solvant aux variations de la température, constituent des inconvénients supplémentaires. Avec ce couplage, la région des acides diaminés est toujours très complexe, d'autant plus que l'acide $\gamma$-aminobutyrique et l'acide $\delta$-aminovalérique viennent également s'y placer. L'arginine n'est pas séparée nettement de la lysine. Au contraire, la méthylhistidine et l'histidine sont mieux isolées du groupe des acides aminés basiques; mais la méthylhistidine occupe une position voisine de la proline. La taurine est bien séparée du groupe sérine–asparagine–glycocolle.

(*b*) *Autres systèmes-solvants.* Dans les couplages butanol–acide acétique + phénol et collidine + phénol, les $R_F$ des acides diaminés sont très nettement différents de ceux des acides dicarboxyliques ($R_F$ bas des acides dicarboxyliques dans tous les solvants; $R_F$ bas des acides diaminés dans le butanol–acide acétique et la collidine, élevés dans le phénol). Dans de nombreux autres types de chromatographie bidimensionnelle, cette dissociation ne s'observe pas et les acides diaminés, les acides dicarboxyliques et leurs amides, les acides aminés soufrés et les acides aminés neutres à chaîne courte sont très proches les uns des autres. Par exemple, le couplage méthanol–eau–pyridine (80:20:4) en première dimension et *tert.*-butanol–méthyléthylcétone–eau–diéthylamine (40:40:20:4) en seconde dimension (REDFIELD[39]) n'est pas applicable à l'étude des milieux biologiques complexes pour cette raison. Pourtant il permet une séparation assez satisfaisante de la méthionine et de la valine. L'acide $\alpha$-aminobutyrique, l'acide $\gamma$-aminobutyrique, l'acide $\beta$-aminoisobutyrique et la méthionine ont des $R_F$ très voisins dans le solvant methanol–eau–pyridine.

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* La collidine peut être remplacée par le système alcool benzylique–acide acétique–eau (50:10:13) (SEN ET BURMA[44]).

La variante technique de BELL et coll.[1] (en première dimension, chromatographie dans le butanol–acide acétique, et en seconde dimension, chromatographie dans le solvant méthanol–eau–pyridine des composés à $R_F$ inférieurs à 0.40 dans le butanol–acide acétique et dans le solvant *tert.*-butanol–méthyléthylcétone–eau–diéthylamine des composés à $R_F$ supérieurs à 0.40) permet une résolution satisfaisante des acides aminés d'un hydrolysat total de protéine (sauf pour l'alanine et la proline), mais elle est difficilement applicable à l'étude des milieux biologiques complexes.

Le couplage *sec.*-butanol–ammoniaque 3% (150:60) en première dimension et *sec.*-butanol–acide formique–eau (150:30:20) en seconde dimension (HAUSSMANN[18]) présente les mêmes inconvénients que celui de REDFIELD[39].

Les systèmes uniphasiques de HARDY et coll.[15]: éthanol–*n*-butanol–acétone–eau–dicyclohexylamine (10:10:5:2), permettent une séparation satisfaisante des acides diaminés, mais ne séparent pas suffisamment le groupe valine–méthionine–tyrosine–leucine–phénylalanine.

En conclusion de cette revue critique, il apparaît que la sélectivité et le pouvoir de résolution des méthodes chromatographiques sont insuffisants pour l'étude précise des milieux biologiques complexes, qui contiennent beaucoup plus de composés donnant une réaction positive à la ninhydrine que les hydrolysats totaux de protéines. Avec tous les couplages chromatographiques décrits, il est relativement facile d'établir la répartition de très nombreux composés: par exemple, certaines cartes indiquent, soit par des points, soit par des cercles, la position de 80 composés "ninhydrine positive"! Quelques expérimentateurs donnent des tableaux des valeurs de $R_F$ avec trois chiffres après le point! Ces données sont trop théoriques et ne peuvent s'appliquer sans risque à une étude pratique, car elles ne tiennent pas compte des problèmes particuliers qui se posent au cours de l'étude de milieux biologiques différents.

## B. PRINCIPE DU COUPLAGE PROPOSÉ

Pour essayer d'obtenir des séparations plus satisfaisantes et d'application facile, nous nous sommes efforcés de mettre au point une technique plus sélective. L'originalité du procédé que nous proposons réside essentiellement dans ***un couplage de méthodes chromatographiques et électrophorétiques classiques***, qui conserve à l'ensemble des manipulations leur simplicité et leur commodité. Son principe peut se schématiser de la façon suivante:

(1) Séparation de base par électrophorèse sur papier en tampon volatil de pH 3.9;

(2) Couplage bidimensionnel électrophorèse sur papier à pH 3.9 et chromatographie butanol–acide acétique–eau pour la séparation des composés "acides" et la séparation des composés "légèrement basiques";

(3) Couplage bidimensionnel électrophorèse sur papier à pH 2.4 et chromatographie butanol–acide acétique–eau pour la séparation des composés "neutres";

(4) Electrophorèse sur papier en tampon volatil de pH 6.5 et de pH 11.7 pour la séparation des composés "basiques".

Les troisième et quatrième étapes peuvent être réalisées à partir du fractionnement de base en électrophorèse sur papier à pH 3.9 grâce à une élution directe et automatique des bandelettes de papier contenant les aminoacides sur une autre feuille d'électrophorèse ou de chromatographie (dispositif d'élution automatique de BISERTE[2]).

Tous les tampons d'électrophorèse et les solvants chromatographiques employés sont facilement éliminés par ventilation d'air froid ou tiède.

## C. APPAREILS ET RÉACTIFS

### 1. *Cuve de chromatographie*

Tous les modèles classiques de cuve peuvent être employés. Les cuves à 4 ou 5 godets, en chlorure de polyvinyle, sont d'un prix de revient modique et leur robustesse est très satisfaisante. La température de la chambre à chromatographie doit être stabilisée à 21° ± 1°.

### 2. *Appareils d'électrophorèse*

Ce sont tous des appareils en V renversé (type DURRUM[9]) entièrement construits en chlorure de polyvinyle (voir Fig. 2). Deux types d'appareils peuvent être utilisés, l'un

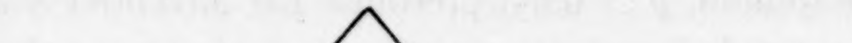

Fig. 2. Appareil d'électrophorèse "en toit" type DURRUM (vue en coupe). La longueur des bacs à tampon est de 68 cm dans les appareils de grandes dimensions et de 32 cm dans les appareils de petites dimensions; les bacs sont indépendants de l'appareil; une électrode en platine est placée directement dans le bac, à l'une de ses extrémités. L'épaisseur des plaques de chlorure de polyvinyle est de 3 mm. La plaque médiane a une épaisseur de 1 cm. A sa partie supérieure, on place une tige de verre qui supporte directement la feuille de papier.

pour des feuilles de 28 × 40 cm, l'autre pour des feuilles de 46 × 57 cm. Il est utile (mais non indispensable) de disposer de plusieurs appareils pour éviter de changer constamment les systèmes-tampons.

### 3. *Redresseur de courant*

Un redresseur de courant robuste permet d'alimenter plusieurs cuves. Le potentiel peut varier entre 0 et 800 V (ou 1000 V), par fractions de 100 V.

### 4. *Dispositif d'élution automatique*

Nous utilisons l'appareil décrit par BISERTE[2]. Le découpage des deux extrémités de la bandelette en pointes, dont l'une est mise au contact de la feuille réceptrice, permet l'élution des acides aminés sous la forme d'une tache de 5 à 6 mm de diamètre. Pour obtenir l'élution sous la forme d'une ligne, pour l'électrophorèse, il suffit de découper la partie inférieure de la bande à éluer pour lui donner la dimension de la ligne de départ désirée. Pour fixer la durée d'élution d'une bandelette de papier, il faut tenir compte de l'épaisseur et de la surface de la bande, de la capacité de la seringue (5, 10 et 20 ml) et de la vitesse de rotation du moteur (1 tour/24 h ou 1 tour/12 h). La variation de vitesse du moteur est facilement obtenue par l'addition d'engrenages supplémentaires. L'élution totale est naturellement contrôlée à la fin de l'opération par pulvérisation de réactif à la ninhydrine sur la bandelette.

### 5. *Formules des tampons*

*Tampon de pH 3.9*: Pyridine–acide acétique–eau (30:100:4870) (MICHL[32], GRASSMANN, HANNIG ET PLÖCKL[14]). Cette formule nous a donné de meilleurs résultats que celle de KICKHÖFEN ET WESTPHAL[25] (tampon pyridine–acide acétique–eau (1:10:89) de pH 3.6). Nous utilisons aussi avec succès la formule pyridine–acide acétique–eau (30:100:3870). Dans ces conditions, la durée de l'électrophorèse peut être raccourcie, car les séparations sont plus rapidement obtenues. Le tampon de pH 3.8 (acide acétique–acétate de sodium, $\mu = 0.05$) préconisé par MONDOVÌ *et al.*[33] permet également un fractionnement de base intéressant, mais il n'est pas volatil.

*Tampon de pH 6.5*: Pyridine–acide acétique–eau (200:8:1792) (HARRIS, SANGER ET NAUGHTON[16]). Ce tampon nous a donné des résultats plus satisfaisants que celui de LOCKHART ET ABRAHAM[30] (tampon collidine–acide acétique–eau (36.3:185:3860) de pH 7).

*Solution d'ammoniaque N* (pH 11.7).

*Solution d'acide acétique N* (pH 2.4).

### 6. *Réactif à la ninhydrine*

Pour les révélations rapides, nous utilisons une solution de ninhydrine à 0.1% dans le *n*-butanol saturé d'eau. Après pulvérisation, les chromatogrammes sont portés à l'étuve à 100° pendant 10 à 15 min.

Pour obtenir une révélation plus satisfaisante, notamment sur le plan quantitatif, nous avons adopté le mode opératoire suivant.

Pour les acides aminés neutres et dicarboxyliques, nous utilisons la formule de BELL et coll.[1]: ninhydrine à 1% dans l'acétone (p/v) contenant 10% (v/v) de tampon phosphate 0.05 $M$ de pH 7. Pour les acides aminés basiques, la solution de ninhydrine est à 1% dans l'acétone (p/v) contenant 6% (v/v) d'acide acétique.

Les chromatogrammes sont trempés complètement dans le réactif à la ninhydrine, égouttés et séchés pendant une heure dans une atmosphère privée d'ammoniac, puis portés à l'étuve à 38° pendant 15 h. Pour une estimation semi-quantitative, les surfaces englobant la totalité de la coloration et des surfaces voisines de mêmes dimensions servant de blanc sont découpées dans le papier et éluées par 3 ml d'acétone contenant 25% d'eau, pendant 20 min sous agitation discontinue. Après centrifugation, les colorations de l'éluat sont lues, dans l'heure qui suit, au spectrophotomètre à 575 m$\mu$ contre un blanc provenant d'un emplacement du chromatogramme le plus voisin possible de la tache à doser (pour l'asparagine, les lectures au spectrophotomètre se font à 350 m$\mu$). Avec la révélation décrite ci-dessus, la proline et l'hydroxyproline forment un complexe violet après le séjour à l'étuve à 38°. Des courbes de référence sont construites à partir de quantités déterminées (0 à 20 $\mu$g) de chaque acide aminé séparé dans les mêmes conditions expérimentales (électrophorèse, chromatographie, etc.). (BISERTE, BOULANGER ET PAYSANT[3].)

## D. FRACTIONNEMENT DE BASE PAR ÉLECTROPHORÈSE À pH 3.9

### 1. *Description de la méthode*

L'électrophorèse à pH 3.9 s'effectue dans un appareil en V renversé, sur du papier Arches 304 (28 × 40 cm), pendant 4 h, sous un potentiel de 300 à 400 V. Le dépôt du mélange des aminoacides à séparer se fait à 12 cm du bord anodique de la feuille préalablement imbibée de tampon, le long d'une ligne de 1.5 à 3 cm, perpendiculaire au grand axe de la feuille. Le volume déposé est habituellement de l'ordre de 10 à 20 $\mu$l.

Après la migration électrophorétique, la feuille est séchée à l'air libre, puis ventilée pendant 15 h ou séchée une demi-heure à l'étuve à 100°, et finalement révélée par pulvérisation ou par trempage dans le réactif à la ninhydrine.

### 2. *Possibilités séparatives*

Les avantages de cette première étape sont nombreux:

le tampon utilisé (pyridine–acide acétique–eau) est entièrement volatil et ne cause donc aucune gêne pour les étapes chromatographiques ultérieures;

cette électrophorèse ne nécessite qu'un appareillage simple et n'exige pas de potentiel élevé;

son pouvoir séparatif est très satisfaisant.

En effet, les acides aminés d'un hydrolysat total de protéine se séparent très facilement en 4 groupes principaux (GRASSMANN, HANNIG ET PLÖCKL[14]): acide aspartique, acide glutamique, le groupe de tous les acides aminés à comportement neutre et le groupe des acides aminés à comportement basique (histidine + lysine + arginine) (Fig. 3).

Après oxydation de l'hydrolysat par l'acide performique, l'acide cystéique formé se sépare également et se localise en avant de l'acide aspartique (Fig. 4).

Dans le cas d'un milieu biologique, la séparation de base est encore plus poussée dès ce premier stade: les acides $\omega$-aminés se situent entre les acides aminés "neutres" et les acides aminés "basiques"; la colamine se localise au delà du groupe des acides aminés "basiques" (Fig. 4).

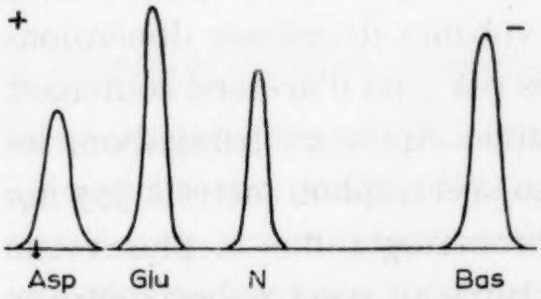

Fig. 3. Electrophorégramme sur papier en tampon acide acétique–pyridine de pH 3.9 (hydrolysat total de protéine). Papier: Arches No. 304; durée: 4 heures sous 320 V; point de départ: 12 cm du côté anodique (la flèche figure le point de dépôt du mélange des acides aminés). Asp: acide aspartique; Glu: acide glutamique; N: acides aminés "neutres"; Bas: acides aminés "basiques" (lysine, arginine, histidine). Les courbes correspondent à l'enregistrement des colorations à la ninhydrine standardisées, à l'aide d'un photodensitomètre équipé d'un filtre vert à 530 m$\mu$. L'enregistrement est réalisé à partir des bandes d'électrophorèse préalablement imbibées par de l'huile de transparence. Le déroulement de la bande se fait mm par mm devant une fente de 1 mm de largeur et de 25 mm de longueur.

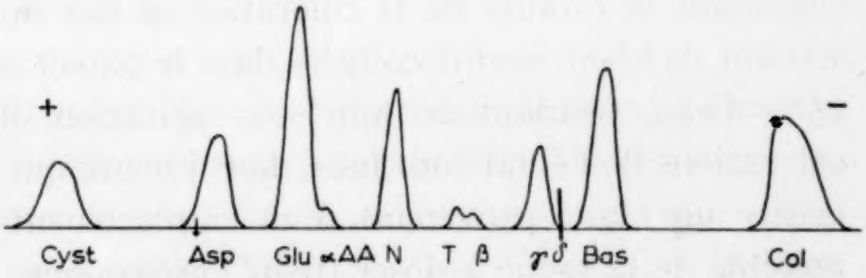

Fig. 4. Electrophorégramme sur papier en tampon acide acétique–pyridine de pH 3.9 (acides aminés libres décelables dans un milieu biologique). Conditions expérimentales identiques à celles décrites dans la Fig. 3. La flèche indique le point de départ de l'électrophorèse. Cyst = acide cystéique; Asp = acide aspartique; Glu = acide glutamique; $\alpha$AA = acide $\alpha$-aminoadipique; N = acides aminés "neutres"; T = acide $\beta$-aminoisobutyrique ou T-spot; $\beta$ = $\beta$-alanine; $\gamma$ = acide $\gamma$-aminobutyrique; $\delta$ = acide $\delta$-aminovalérique; Bas = acides aminés basiques (arginine, lysine, méthylhistidine, histidine, ornithine); Col = colamine.

Bien souvent, l'aspect de l'électrophorégramme est encore plus complexe. Afin de préciser davantage les possibilités de cette méthode, nous avons étudié le comportement de nombreux dérivés "ninhydrine positive" ou non, naturels ou synthétiques, dans les diverses zones de l'électrophorégramme à pH 3.9.

### (*a*) *Zone des composés à comportement acide* (Figs. 5 et 6)

(*i*) *Composés "ninhydrine positive"*. Dans toute la région de l'électrophorégramme située en avant du groupe des acides aminés "neutres", on peut trouver un certain nombre de composés "ninhydrine positive" tels que l'*acide homocystéique* qui se sépare facilement de l'acide cystéique, le *tyrosine-O-sulfate* qui se situe juste en avant de l'acide aspartique, le *glutathion traité par l'acide performique* (G-SH → G-SO$_3$H) qui se situe entre l'acide cystéique et l'acide aspartique, le *glutathion réduit* (G-SH) et *oxydé* (G-S-S-G) qui sont placés entre l'acide aspartique et l'acide glutamique. Le glutathion oxydé est légèrement plus rapide que le glutathion réduit (Fig. 6). Toutefois pour séparer ces deux composés, il faut réaliser un couplage électrophorèse à pH 3.9

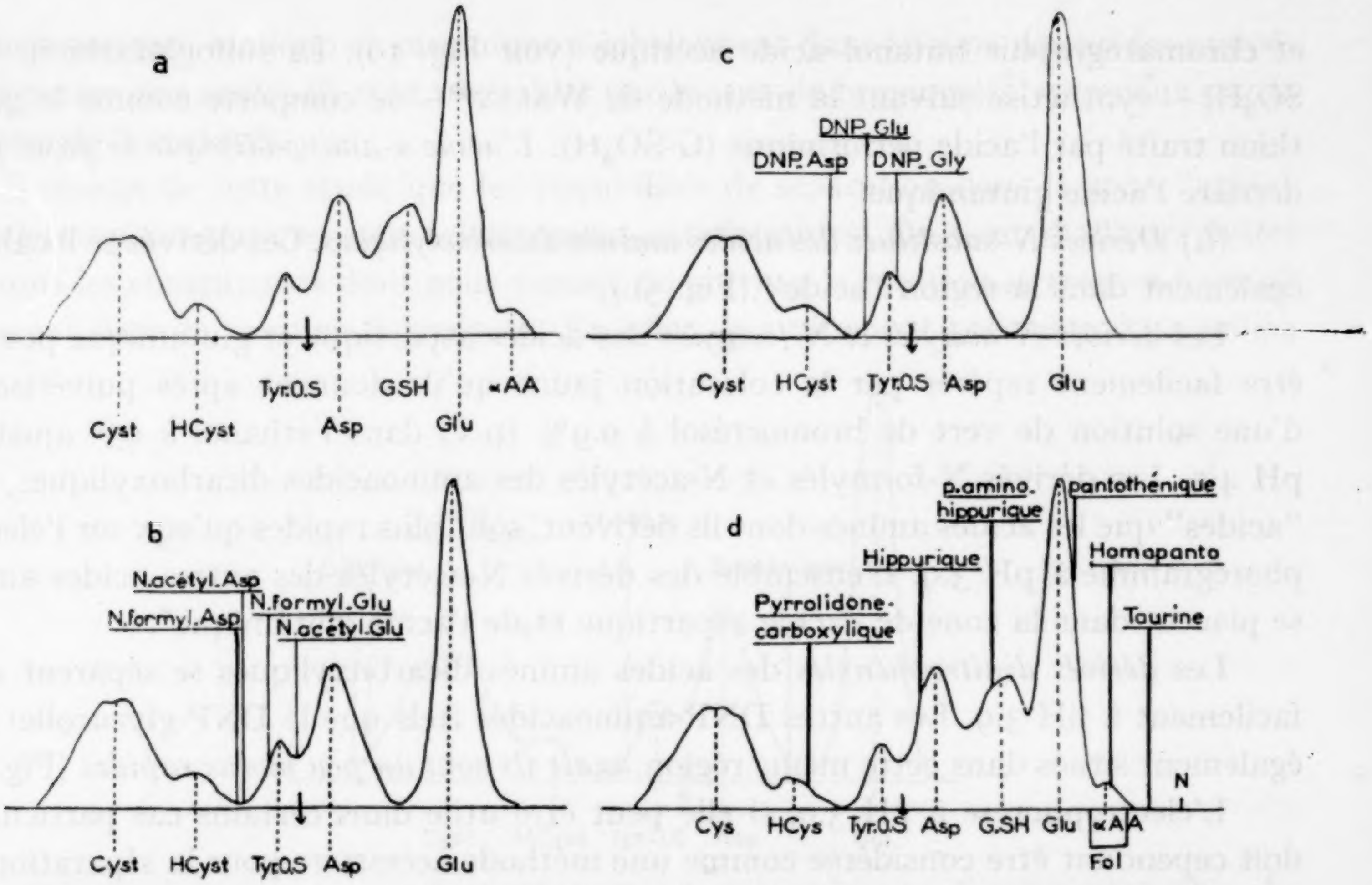

Fig. 5. Electrophorégramme sur papier à pH 3.9 des "composés à comportement acide" (4 h, 320 V, point de départ à 12 cm du côté anodique). (a) Comportement des composés "ninhydrine positive". Cyst = acide cystéique; HCyst = acide homocystéique; Tyr-O-S = tyrosine-O-sulfate; Asp = acide aspartique; GSH = glutathion réduit; Glu = acide glutamique; αAA = acide α-aminoadipique. (b) Comportement des dérivés N-acétylés et N-formylés de l'acide aspartique et de l'acide glutamique (révélation au vert de bromocrésol). (c) Comportement des dérivés dinitrophénylés de l'acide aspartique, de l'acide glutamique et du glycocolle (dérivés colorés en jaune et possédant une fluorescence brune en lumière de Wood). (d) Comportement des formes combinées d'acides aminés ou des dérivés d'acides aminés à comportement acide. Acide pyrrolidone-carboxylique: révélation par la méthode de Rydon et Smith[40]; acides hippurique et *p*-aminohippurique: révélation au *p*-diméthylaminobenzaldéhyde en solution dans l'anhydride acétique; acide pantothénique et homopantothénique: révélation par la ninhydrine après hydrolyse partielle par pulvérisation d'acide acétique; taurine et N (acides aminés neutres): révélation à la ninhydrine; Fol = acide folique: localisation après élution des bandes et hydrolyse totale suivie d'une identification de l'acide glutamique libéré, par électrophorèse ou chromatographie.

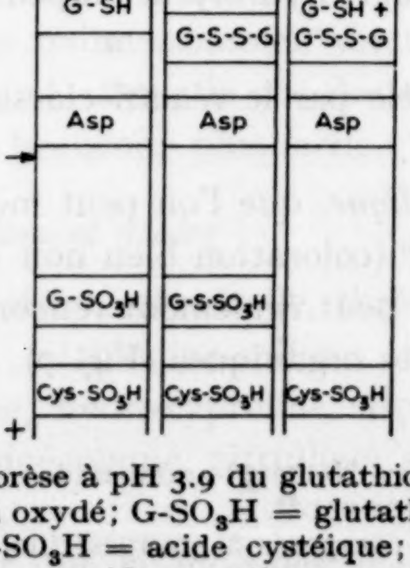

Fig. 6. Comportement en électrophorèse à pH 3.9 du glutathion et de ses dérivés. G-SH = glutathion réduit; G-S-S-G = glutathion oxydé; G-SO$_3$H = glutathion traité par l'acide performique; G-S-SO$_3$H = sulfoglutathion; Cys-SO$_3$H = acide cystéique; Glu = acide glutamique; Asp = acide aspartique.

et chromatographie butanol–acide acétique (voir Fig. 10). Le sulfoglutathion (G-S-$SO_3H$) — synthétisé suivant la méthode de WALEY[48] — se comporte comme le glutathion traité par l'acide performique (G-$SO_3H$). *L'acide α-aminoadipique* se situe juste derrière l'acide glutamique.

(*ii*) *Dérivés N-substitués des acides aminés dicarboxyliques.* Ces dérivés se localisent également dans la région "acide" (Fig. 5b).

Les *dérivés N-acétylés et N-formylés* des acides aspartique et glutamique peuvent être facilement repérés par la coloration jaune qu'ils donnent après pulvérisation d'une solution de vert de bromocrésol à 0.9% (p/v) dans l'éthanol à 95$^c$ ajustée à pH 4.5. Les dérivés N-formylés et N-acétylés des aminoacides dicarboxyliques, plus "acides" que les acides aminés dont ils dérivent, sont plus rapides qu'eux sur l'électrophorégramme à pH 3.9. L'ensemble des dérivés N-acétylés des autres acides aminés se placent dans la zone de l'acide aspartique et de l'acide glutamique*.

Les *dérivés dinitrophénylés* des acides aminés dicarboxyliques se séparent assez facilement à pH 3.9. Les autres DNP-aminoacides (tels que le DNP-glycocolle) sont également situés dans cette même région, *mais ils sont un peu moins rapides* (Fig. 5c).

L'électrophorèse à pH 3.9, si elle peut être utile dans certains cas particuliers, doit cependant être considérée comme une méthode accessoire pour la séparation des dérivés N-substitués des acides aminés.

(*iii*) *Formes combinées d'aminoacides.* Certaines formes combinées d'acides aminés, ainsi que certains dérivés "ninhydrine négative", ont également un comportement "acide". Ce sont principalement (Fig. 5d):

*l'acide hippurique* et *l'acide p-aminohippurique*, qui peuvent être révélés spécifiquement par une solution de *p*-diméthylaminobenzaldéhyde à 4% (p/v) dans l'anhydride acétique;

*l'acide pantothénique* et *l'acide homopantothénique*, qui sont identifiés après hydrolyse partielle; celle-ci peut être obtenue par 2 pulvérisations successives d'acide acétique à 50%; après chauffage, la *β*-alanine et l'acide *γ*-aminobutyrique libérés sont révélables par la ninhydrine (BISERTE, PLAQUET ET BOULANGER[4]);

*l'acide folique*; l'identification de ce composé est conduite de la façon suivante: après élution de la zone contenant l'acide folique, l'éluat est hydrolysé totalement par l'acide chlorhydrique 5.6 *N*; dans l'hydrolysat on peut mettre facilement en évidence l'acide glutamique;

le *coenzyme A*, qui se révèle par le réactif classique au nitroprussiate, caractéristique des groupements SH**.

*l'acide pyrrolidone-carboxylique*, que l'on peut mettre en évidence à l'aide de la réaction de RYDON ET SMITH[40] (coloration bleu noir sur fond légèrement bleu).

(*iv*) *Acides organiques.* On peut également rencontrer dans la région "acide" de l'électrophorégramme des acides organiques (Fig. 7). Les acides fumarique, citrique, α-cétoglutarique, malique et succinique s'échelonnent dans la zone des acides aminés à comportement acide; ils sont révélables par le vert de bromocrésol ou mieux par la réaction de Saarnio[41].

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* Le comportement de nombreux N-acétyl-aminoacides a été étudié en chromatographie et électrophorèse sur papier par WHITEHEAD[49].

** Pour la chromatographie des composés à groupes thiol, notamment le CoA, voir PRICE ET CAMPBELL[38].

Il résulte de cette étude que les possibilités de séparation dans la zone "acide" de l'électrophorégramme sont relativement satisfaisantes. On peut d'ailleurs éviter que tous les constituants dont nous venons de préciser la position se trouvent simultanément dans la solution à analyser. En effet, le traitement préalable d'un milieu biologique par une résine échangeuse de cations fortement acide (Amberlite IR 120 ou Dowex 50) permet de simplifier le problème. Les acides aspartique, glutamique, α-aminoadipique et le glutathion sont fixés, tandis que des composés "ninhydrine positive", comme le tyrosine-O-sulfate, ou "ninhydrine négative", comme l'acide folique, les dérivés N-formylés et N-acétylés des acides dicarboxyliques, les acides pantothénique et homopantothénique, les acides hippurique et *p*-aminohippurique, *ne sont pas* ou sont *peu* retenus sur les résines à échange de cations.

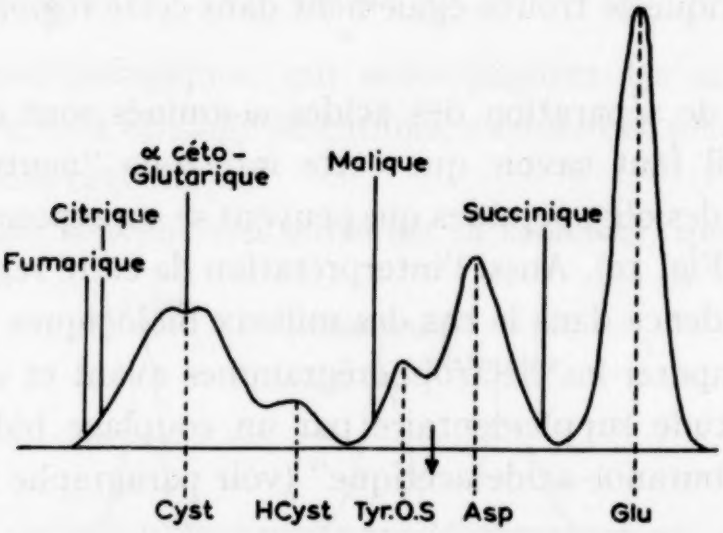

Fig. 7. Electrophorégramme sur papier à pH 3.9 d'acides organiques. Les lignes verticales figurent l'emplacement d'acides organiques par rapport aux acides aminés à comportement acide (révélation des acides organiques par la méthode de Saarnio[41] à l'aide d'un réactif aniline–xylose).

En conclusion, l'interprétation de cette zone de l'électrophorégramme doit toujours être très prudente dans le cas d'un milieu biologique non hydrolysé, d'autant plus qu'en dehors des composés cités plus haut, des oligopeptides peuvent se localiser également dans cette zone (voir Fig. 10). Il est indispensable de compléter l'étude de cette zone acide par un couplage bidimensionnel "électrophorèse à pH 3.9 et chromatographie butanol–acide acétique". Dans le cas des milieux biologiques hydrolysés totalement, l'interprétation est beaucoup plus facile.

### (*b*) *Zone des composés à comportement neutre*

Les acides aminés "neutres" (sérine, glycocolle, thréonine, alanine, acide α-aminobutyrique, valine, méthionine, méthionine-sulfone, méthionine-sulfoxyde, leucines, phénylalanine, tyrosine, proline, hydroxyproline, asparagine, glutamine, sarcosine, cystine, tryptophanne, acide pipécolique, citrulline) se localisent dans cette zone à comportement neutre à pH 3.9.

Il est important de savoir que la zone des acides aminés "neutres" contient également le glucose, l'urée et la créatine. Au cours de cette électrophorèse à faible potentiel il n'y a pas de fractionnement de l'ensemble des composés à comportement neutre.

(*c*) *Zone des acides $\omega$-aminés* (Fig. 4)

L'acide $\beta$-aminoisobutyrique ou T-spot, la $\beta$-alanine, l'acide $\gamma$-aminobutyrique se séparent facilement à pH 3.9 et se localisent dans l'interzone "neutres-basiques". L'acide $\delta$-aminovalérique se trouve également dans cette région, au niveau de l'acide $\gamma$-aminobutyrique*.

Les possibilités de séparation des acides $\omega$-aminés sont donc particulièrement intéressantes. Mais il faut savoir que cette interzone "neutres-basiques" est très souvent occupée par des oligopeptides qui peuvent se superposer aux acides $\omega$-aminés et les masquer (voir Fig. 10). Aussi l'interprétation de cette région doit-elle être faite avec une grande prudence dans le cas des milieux biologiques non hydrolysés; il est indispensable de comparer les électrophorégrammes avant et après hydrolyse totale et d'effectuer une étude supplémentaire par un couplage bidimensionnel "électrophorèse à pH 3.9 et butanol–acide acétique" (voir paragraphe E).

(*d*) *Zone des composés à comportement basique* (Fig. 4)

À pH 3.9, l'arginine, l'ornithine, la lysine, l'histidine et la méthylhistidine ne se séparent pas l'une de l'autre. Seul de tous les acides diaminés, l'acide $\alpha,\gamma$-diaminobutyrique se distingue assez nettement, car il migre davantage vers la cathode (Fig. 8). Au contraire, le dipeptide *carnosine* ($\beta$-alanylhistidine) est un peu moins rapide

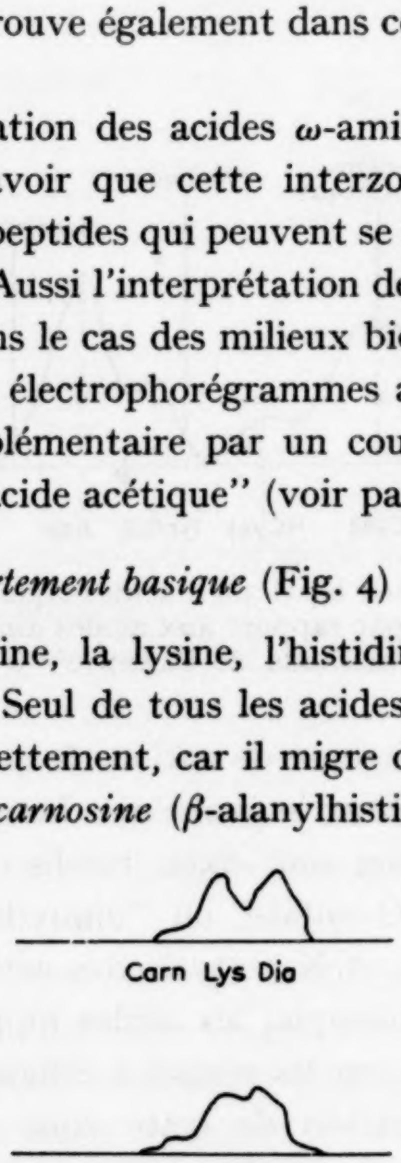

Fig. 8. Electrophorèse à pH 3.9 des composés "ninhydrine positive" à "comportement basique" (4 h, 350 V, point de départ à 9 cm du côté anodique; distance parcourue par la lysine à partir du point de départ: 16.3 cm). Lys = lysine (l'arginine, l'ornithine, l'histidine et la méthylhistidine ont un comportement identique à celui de la lysine); Dia = acide $\alpha$, $\gamma$-diaminobutyrique; Carn = carnosine; Tyr = tyramine; Glucosamine: la glucosamine se comporte sensiblement comme la carnosine.

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* Après élution de cette zone, on peut réaliser une chromatographie bidimensionnelle classique (butanol–acide acétique et phénol ($NH_3$)). En plaçant des témoins latéraux et un témoin interne de valine, il est facile de distinguer l'acide $\gamma$-aminobutyrique et l'acide $\delta$-aminovalérique qui se situent au voisinage de la valine.

Solvant butanol–acide acétique–eau (4 : 1 : 5): $R_F$ acide $\gamma$-aminobutyrique = 0.43, $R_F$ acide $\delta$-aminovalérique = 0.49.

Solvant phénol ($NH_3$): $R_F$ acide $\gamma$-aminobutyrique = 0.77, $R_F$ acide $\delta$-aminovalérique = 0.79.

que les acides aminés basiques. Dans les extraits de muscle, sa présence se traduit par un relèvement de la pente ascendante du sommet correspondant aux acides aminés basiques. Cet accident disparaît sur les électrophorégrammes des hydrolysats totaux de ces extraits.

La *tyramine* est la seule amine biologique que l'on peut rencontrer dans ce groupe des composés à comportement basique. On y trouve également la *glucosamine* (Fig. 8).

### (*e*) *Zone des amines*

La plupart des amines biologiques, qui accompagnent les acides aminés dans le fractionnement sur résines à échange de cations, viennent se placer *au delà* des acides aminés basiques, du côté cathodique.

Le composé le plus souvent rencontré est la *colamine*, que l'on peut identifier facilement à l'aide d'un témoin latéral. Il faut toutefois signaler que cette zone contient fréquemment d'autres composés colorables à la ninhydrine; la majorité des amines biologiques (cadavérine, putrescine, agmatine, allylamine, histamine) viennent se placer dans cette région et ne peuvent être séparés efficacement par cette méthode (Fig. 9)*.

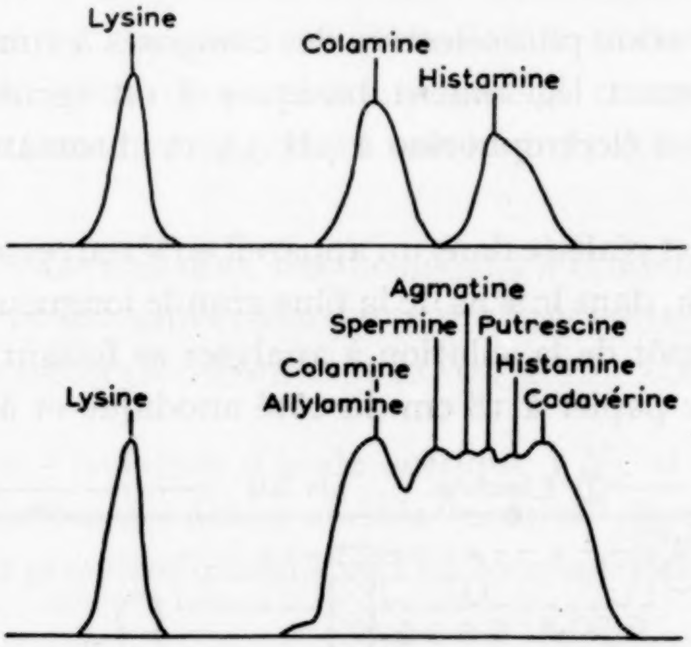

Fig. 9. Electrophorèse à pH 3.9 des amines biologiques (320 V, 210 min, point de départ: 8 cm du côté anodique, révélation à la ninhydrine; distance parcourue par la lysine à partir du point de départ: 16 cm). La majorité des amines viennent se placer au delà de la colamine du côté cathodique. Leur séparation n'est pas possible. La colamine est le composé le plus fréquemment rencontré. La tyramine se comporte comme les acides aminés basiques.

La *β-mercaptoéthylamine* (révélation à la ninhydrine et révélation au nitroprussiate de sodium) se rencontre également dans cette zone de l'électrophorégramme, ainsi que la *spermine*.

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* Le comportement chromatographique de ces amines est lui aussi très particulier. Dans le système phénol($NH_3$), elles forment des traînées allongées dont le $R_F$ est voisin de 1. Le butanol–acide acétique permet la séparation de la tyramine et de la colamine. Si l'on donne arbitrairement la valeur de 1 au $R_F$ de la tyramine (amine la plus rapide), le $R_F$ de la colamine est de 0.59. FISCHER ET BOHN[10] effectuent la séparation des amines en électrophorèse sur papier en tampon pyridine–acide acétique–acide citrique. GÄYER[11] utilise l'électrophorèse à haut potentiel. Une séparation partielle des amines par électrophorèse sur papier (à pH 4 et à pH 6.5) et par chromatographie sur papier a été décrite également par HERBST[19]. KAPELLER-ADLER ET IGGO[24] ont séparé l'histamine et ses dérivés N-méthylés par chromatographie sur papier et LOCKHART[29] a étudié le comportement chromatographique des dérivés dinitrophénylés des amines.

En conclusion, l'électrophorèse à pH 3.9 constitue une étape de fractionnement de base très intéressante. Quant aux composés dont ce premier stade n'a pu réaliser la résolution, c'est-à-dire principalement le groupe des acides aminés "neutres" et celui des acides aminés "basiques", ils doivent être obligatoirement soumis à d'autres techniques séparatives après élution automatique quantitative.

### E. IDENTIFICATION PARTICULIÈRE DES COMPOSÉS À COMPORTEMENT ACIDE ET DES COMPOSÉS À COMPORTEMENT LÉGÈREMENT BASIQUE (COUPLAGE BIDIMENSIONNEL ÉLECTROPHORÈSE À pH 3.9 ET CHROMATOGRAPHIE BUTANOL–ACIDE ACÉTIQUE)

Pour obtenir une séparation plus sélective des composés à comportement acide et des composés à comportement légèrement basique, il est recommandé d'effectuer un couplage bidimensionnel électrophorèse à pH 3.9 et chromatographie butanol–acide acétique (Fig. 10).

L'électrophorèse est réalisée dans un appareil en V renversé de grandes dimensions, sous 600 V, pendant 8 h, dans le sens de la plus grande longueur d'une feuille de papier Whatman No. 1, le dépôt de la solution à analyser se faisant en un point situé dans un coin de la feuille de papier à 18 cm du côté anodique et à 10 cm de l'autre bord.

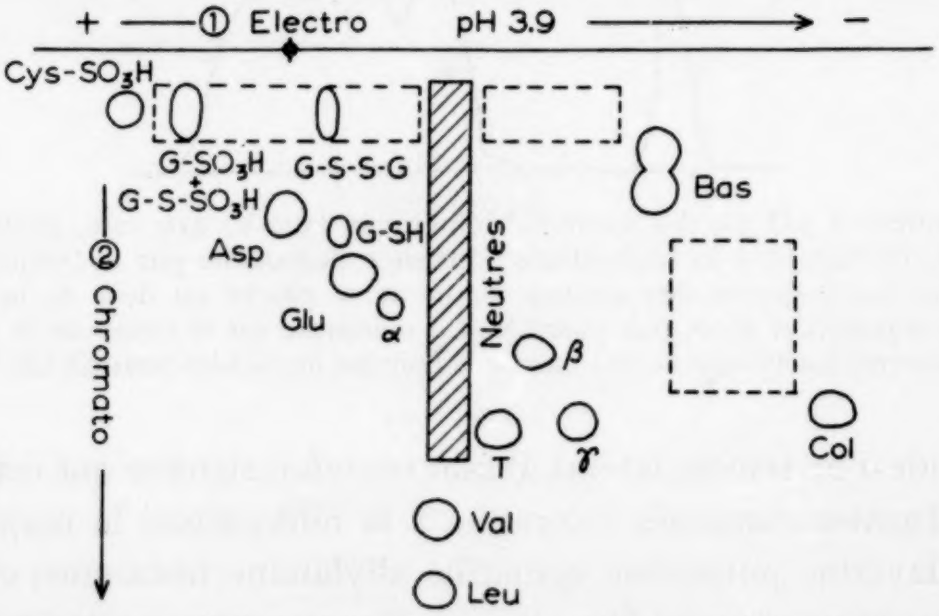

Fig. 10. Séparation des composés acides et des composés légèrement basiques (couplage bidimensionnel comportant une électrophorèse à pH 3.9 sur une feuille de papier Whatman No. 1 (57 × 47 cm) en première dimension et une chromatographie dans le solvant butanol–acide acétique–eau (4 : 1 : 5) en seconde dimension). Les abréviations suivantes ont été utilisées: Cys-SO$_3$H = acide cystéique; Asp = acide aspartique; G-SH = glutathion réduit; G-S-S-G = glutathion oxydé; G-SO$_3$H = glutathion traité par l'acide performique; G-S-SO$_3$H = sulfoglutathion; Glu = acide glutamique; $\alpha$ = acide $\alpha$-aminoadipique; Val = valine; Leu = leucines; Neutres = ensemble des acides aminés neutres; T = acide $\beta$-aminoisobutyrique ou T-spot; $\beta$ = $\beta$-alanine; $\gamma$ = acide $\gamma$-aminobutyrique; Bas = acides aminés à comportement basique (ornithine, lysine, arginine, histidine, méthylhistidine) + glucosamine + carnosine + ansérine; Col = colamine. Dans la zone des acides aminés neutres, des ébauches de séparation peuvent être observées, de même que dans la zone des composés basiques. Les rectangles limités par des lignes pointillées correspondent aux zones où l'on rencontre le plus souvent, au cours de l'étude des milieux biologiques complexes, de nombreux composés "ninhydrine positive" qui ne correspondent pas à des acides aminés.

Après séchage sous une hotte par ventilation d'air froid pendant 16 h, la chromatographie descendante dans le système classique *n*-butanol–acide acétique–eau (4:1:5) est effectuée pendant 24 à 36 h.

Après révélation, on obtient dans la zone des composés acides une séparation très satisfaisante de l'acide cystéique, de l'acide aspartique, des différentes formes du glutathion, de l'acide glutamique, de l'acide $\alpha$-aminoadipique. Dans les milieux biologiques non hydrolysés, on observe également de nombreux oligopeptides dans cette région du papier (Fig. 10). Dans l'interzone "composés neutres–composés basiques", l'identification de l'acide $\beta$-aminoisobutyrique, de la $\beta$-alanine et de l'acide $\gamma$-aminobutyrique est très aisée. La séparation des acides aminés basiques et celle des composés à comportement neutre sont insuffisantes. Ce couplage est particulièrement intéressant pour étudier les milieux biologiques non hydrolysés (voir BISERTE, BOULANGER ET PAYSANT[3]). La présence en différentes régions du chromatogramme d'oligopeptides ou d'autres composés réagissant à la ninhydrine et distincts des acides aminés habituels, peut être confirmée par la comparaison des couplages bidimensionnels des fractions hydrolysées et non hydrolysées.

### F. IDENTIFICATION PARTICULIÈRE DES COMPOSÉS À COMPORTEMENT NEUTRE (COUPLAGE BIDIMENSIONNEL ÉLECTROPHORÈSE À pH 2.4 ET CHROMATOGRAPHIE BUTANOL–ACIDE ACÉTIQUE–EAU)

L'électrophorèse à pH 2.4 (solution d'acide acétique 1 *N*), si elle est par elle-même insuffisante pour la résolution des acides aminés "neutres", peut être utilisée de façon très satisfaisante comme première dimension d'un couplage électrophorèse–chromatographie.

Elle est réalisée dans un appareil en V renversé de grandes dimensions, sous 400 V, pendant 18 h, dans le sens de la plus grande dimension d'une feuille de papier Whatman No. 3 (46 × 57 cm), le point de départ se trouvant à 8 cm du bord anodique et à 10 cm de l'autre bord. Le dépôt des acides aminés se fait par élution automatique à partir de la bandelette découpée sur un électrophorégramme à pH 3.9. L'appareil d'élution est équipé avec un moteur de 1 tour/12 h et une seringue de 20 ml; l'opération est prolongée pendant 45 min.

Après séchage de la feuille, cette électrophorèse est suivie d'une chromatographie unidimensionnelle descendante dans le solvant butanol–acide acétique–eau (4:1:5), effectuée dans le sens perpendiculaire à la migration électrophorétique, et dont la durée est de 20 h. Le chromatogramme est séché soit à l'étuve à 100°, soit, si l'on désire effectuer une chromatographie quantitative, à la température ordinaire pendant 2 jours par ventilation d'air froid dans une atmosphère exempte d'ammoniac. La révélation est réalisée de préférence par la solution de ninhydrine à 1% (voir paragraphe C).

Dans ces conditions, la résolution des acides aminés "neutres" est très satisfaisante (Fig. 11). C'est ainsi que la sérine et le glycocolle, la méthionine et la valine, la phénylalanine et les leucines sont nettement séparées, ainsi que le tryptophanne.

La méthionine-sulfone se place au voisinage de l'hydroxyproline; mais l'identification de celle-ci ne pose pas de problème particulier du fait de la coloration orange qu'elle donne à la révélation à la ninhydrine*; elle peut d'ailleurs être révélée également à l'aide de réactions spécifiques, notamment celle de JEPSON ET SMITH[22]. La distinction entre l'asparagine et la glutamine est également facilitée par des différences de coloration (glutamine: violette; asparagine: brune). La citrulline se place au même endroit que la glutamine. Après hydrolyse totale du milieu biologique, la persistance

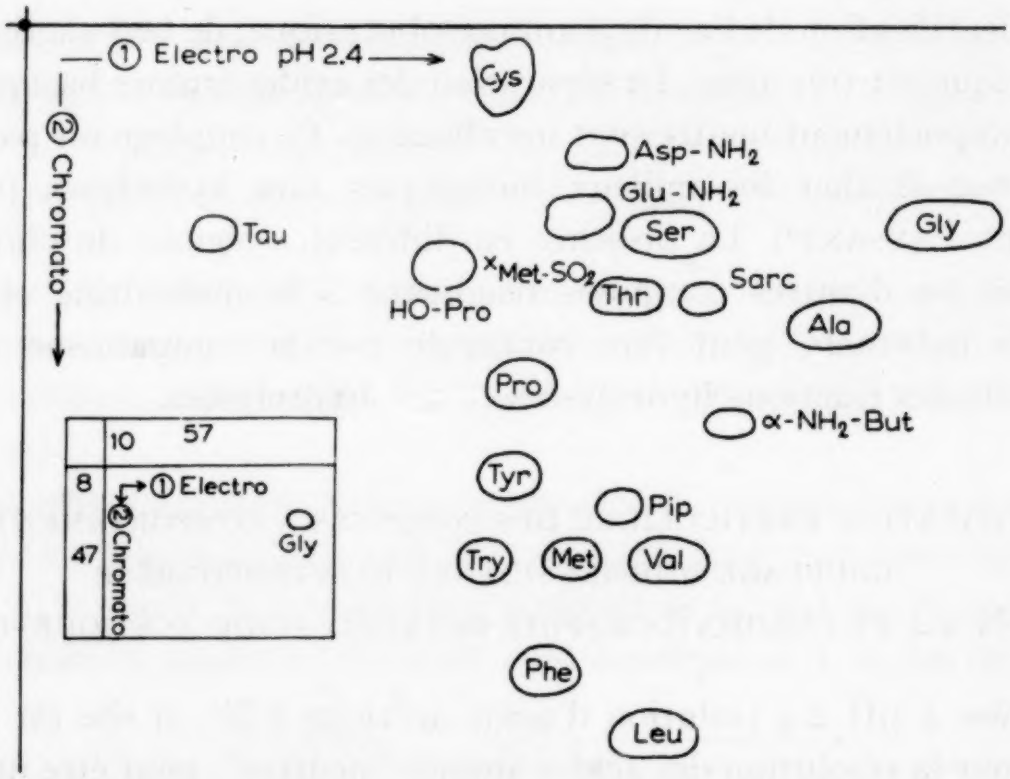

Fig. 11. Séparation des acides aminés "neutres" (electrophorèse à pH 2.4 suivie d'une chromatographie unidimensionnelle dans le solvant butanol–acide acétique–eau (4 : 1 : 5). *1ère opération:* Electrophorèse dans le sens de la plus grande dimension (57 cm) d'une feuille de papier Whatman No. 3 dans un appareil type DURRUM (400 V, 18 h). Le point de dépôt (point noir sur le schéma) se trouve à 8 cm du bord de la feuille du côté anodique et à 10 cm de l'autre bord. Après 18 h de passage du courant, le glycocolle se trouve à 43 cm du point d'origine. *2ème opération:* Chromatographie unidimensionnelle dans le solvant butanol–acide acétique–eau (4 : 1 : 5) pendant 20 h (ou davantage). À la partie inférieure gauche de la figure, nous avons représenté la position du point d'origine et la disposition générale de l'opération. Gly indique la position du glycocolle à la fin du couplage. Les abréviations suivantes ont été utilisées: Tau = taurine; Cys = cystine; Asp-NH$_2$ = asparagine; Glu-NH$_2$ = glutamine; Ser = sérine; Gly = glycocolle; HO–Pro = hydroxyproline; Met-SO$_2$ = méthionine-sulfone; Thr = thréonine; Sarc = sarcosine; Pro = proline; $\alpha$-NH$_2$-But = acide $\alpha$-aminobutyrique; Tyr = tyrosine; Pip = acide pipécolique; Try = tryptophanne; Met = méthionine; Val = valine; Phe = phénylalanine; Leu = leucines. Les positions de la sarcosine, de la proline, de l'hydroxyproline, de l'acide pipécolique, de la citrulline (qui occupe la même position que la glutamine) et du tryptophanne peuvent être confirmées par des réactions spécifiques. L'asparagine (coloration brune) et la glutamine disparaissent à l'hydrolyse totale. La présence de méthionine-sulfone doit être confirmée à l'aide d'un témoin interne. Dans les milieux biologiques, de nombreux autres composés "ninhydrine positive" peuvent se rencontrer sur la carte.

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* Cette coloration orange est observée *au début* de la révélation avec le réactif à la ninhydrine concentré; après le séjour à l'étuve à 38°, l'hydroxyproline présente la coloration violet-pourpre classique (pour le mécanisme de formation du complexe pourpre, voir JOHNSON ET MCCALDIN[23]).

La séparation des acides aminés cycliques (hydroxyproline, *allo*hydroxyproline, proline, baikiaïne, acide pipécolique, acide 5-hydroxypipécolique, acide *allo*-5-hydroxypipécolique) peut être réalisée par une chromatographie bidimensionnelle suivant la méthode de PIEZ, IRREVERRE ET WOLFF[37] (première dimension: alcool butylique tertiaire–acide formique à 88 %–eau (70 : 15 : 15) (v/v), 18 h sur papier Schleicher et Schüll No. 598; seconde dimension: alcool amylique tertiaire–2,4-lutidine–eau (178 : 178 : 114) (v/v), 18 à 22 h).

d'une tache ninhydrine positive dans cette zone rend nécessaire la réalisation d'une révélation spécifique de la citrulline. Le seul problème qui ne soit pas résolu est celui de la séparation leucine–isoleucine; mais celle-ci peut être facilement obtenue par l'utilisation du système-solvant de HÖGSTRÖM[20] (méthyléthylcétone–acétone–eau (3:1:0.6)): on peut le mettre en œuvre soit en chromatographie unidimensionnelle descendante pendant 30 h, après avoir préalablement séparé les acides aminés "neutres" en électrophorèse à pH 3.9 et élué automatiquement la zone correspondante sur une bande de papier Whatman No. 1; soit en un couplage bidimensionnel "électrophorèse à pH 2.4 et chromatographie", en partant de cette même zone des acides aminés neutres*. Les $R_F$ calculés par rapport à celui du tryptophanne sont les suivants: isoleucine: $R_F$ (Try) 0.78; leucine: $R_F$ (Try) 0.86.

Dans les milieux biologiques complexes, de nombreuses taches "ninhydrine positive" qui ne correspondent pas à des acides aminés neutres peuvent être decelées sur le papier. L'interprétation de la zone délimitée par la cystine, l'hydroxyproline et la glutamine est souvent délicate.

## G. IDENTIFICATION PARTICULIÈRE DES COMPOSÉS À COMPORTEMENT BASIQUE

### 1. *Chromatographie bidimensionnelle*

Après élution, à partir d'un électrophorégramme à pH 3.9, de la bande des acides aminés basiques, l'étude de ces composés peut être abordée par la chromatographie bidimensionnelle classique (butanol–acide acétique et phénol ($NH_3$)).

L'identification de la glucosamine, de la tyramine, éventuellement de l'acide $\gamma$-aminobutyrique, est facile. La zone des acides aminés basiques est déjà nettement moins confuse que sur le chromatogramme bidimensionnel obtenu à partir du mélange initial qui n'a pas été "simplifié" par électrophorèse à pH 3.9. Toutefois, on ne peut obtenir de cette façon des résultats précis**.

### 2. *Électrophorèse dans l'ammoniaque 1 N (pH 11.7)*

La séparation de l'histidine, de la lysine et de l'arginine est facilement réalisée par une électrophorèse à pH 11.7 (solution d'ammoniaque 1 *N*) dans un appareil en V renversé, sur du papier Whatman No. 1, sous un potentiel de 400 V, pendant 15 h, la ligne de départ étant placée à 11 cm du bord cathodique de la feuille***. Le dépôt des acides aminés se fait par élution automatique à partir de la bandelette qui contient les composés basiques et qui a été découpée sur un électrophorégramme à pH 3.9. Il est indispensable de placer des témoins latéraux.

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* Cette chromatographie doit être effectuée dans des cuves en verre, car les cuves en chlorure de polyvinyle se déforment en présence de ce système-solvant.

** La séparation des acides aminés basiques sur un papier imprégné par une résine à échange de cations (28% d'Amberlite IR 120) est relativement satisfaisante (TUCKERMANN[45]).

*** Le tampon de pH 11.6 (phosphate disodique–soude) utilisé par GERLAXHE, CASIMIR ET RENARD[12] a été utilisé également pour la séparation des acides aminés basiques. Il a le désavantage de ne pas être volatil. HARRIS ET WARREN[17] ont également réalisé l'électrophorèse de ces composés à pH 11.5 (tampon $CO_3Na_2$ 0.05 *M*).

Les possibilités séparatives de ce procédé sont nettement meilleures que celles des méthodes chromatographiques. Il permet en effet d'identifier aisément, en présence de témoins latéraux, l'arginine, la lysine et l'ornithine (Fig. 12).

Toutefois, dans le cas des milieux biologiques, le problème est beaucoup plus complexe: la zone des acides aminés "basiques" à pH 3.9 peut contenir, outre l'arginine, la lysine et l'ornithine, de l'histidine, de la méthylhistidine, de la glucosamine, de la tyramine*, des peptides à caractère basique tels que la carnosine et l'ansérine; elle peut comporter éventuellement de l'acide $\gamma$-aminobutyrique provenant d'une récupération trop large de la bande des acides aminés basiques.

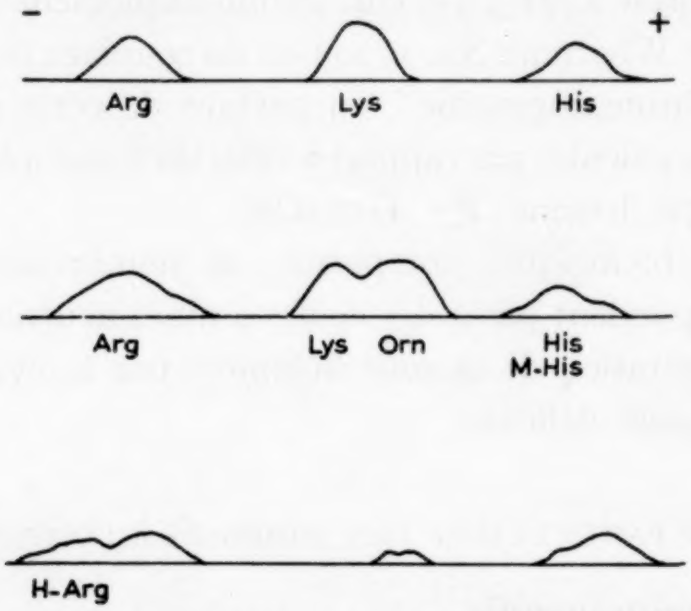

Fig. 12. Electrophorèse à pH 11.7 ($NH_4OH$ 1 *N*) des acides aminés basiques (papier Whatman No. 1, départ à 11 cm de la cathode, 400 V pendant 15 h, dans un appareil type DURRUM). Arg = arginine (distance parcourue à partir du point de départ: 4 cm); Lys = lysine (distance parcourue à partir du point de départ: 9.5 cm); Orn = ornithine (distance parcourue à partir du point de départ: 11.5); His = histidine; M-His = méthylhistidine (distance parcourue à partir du point de départ: 16 cm); la carnosine ne se sépare pas du groupe histidine–méthylhistidine; H-Arg = homoarginine (distance parcourue à partir du point de départ: 3 cm). *En haut:* Séparation du mélange arginine–lysine–histidine (cas d'un hydrolysat total). *Au milieu:* Séparation du mélange arginine–lysine–ornithine–histidine et méthylhistidine (cas fréquent d'un mélange d'acides aminés basiques d'un milieu biologique). *En bas:* séparation du mélange homoarginine–arginine–lysine–histidine (cas d'une protéine partiellement guanidylée où la lysine est transformée en homoarginine).

La présence éventuelle de la glucosamine et de la tyramine peut être déterminée par une chromatographie bidimensionnelle classique (voir plus haut paragraphe G 1). Mais de toute façon l'électrophorèse à pH 11.7 ne permet pas de séparer l'histidine de la méthylhistidine.

### 3. *Électrophorèse à pH 6.5*

Le problème de la séparation de l'histidine et de la méthylhistidine peut être résolu par une électrophorèse pratiquée en tampon volatil de pH 6.5 (pyridine–acide acétique–eau (200:8:1792)) dans un appareil en V renversé de grandes dimensions, sur du papier Whatman No. 1, pendant 15 h, sous un potentiel de 400 V, le dépôt étant effectué à 28.5 cm du bord cathodique de la feuille et le papier étant placé de façon dissymétrique avec un côté cathodique plus long (30.5 cm) que le côté anodique.

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* La glucosamine peut empiéter sur l'arginine, et la tyramine sur la lysine.

Dans ces conditions, la séparation de l'histidine et de la méthylhistidine est satisfaisante (Fig. 13). Dans les hydrolysats totaux, elle peut donc s'appliquer utilement à l'identification et au dosage de ces acides aminés.

Dans certains milieux biologiques non hydrolysés (muscle, urine par exemple), la séparation est cependant plus délicate du fait de la présence de la carnosine (β-alanyl-histidine) et de l'ansérine (β-alanyl-méthylhistidine) qui sont plus ou moins confondues avec la méthylhistidine.

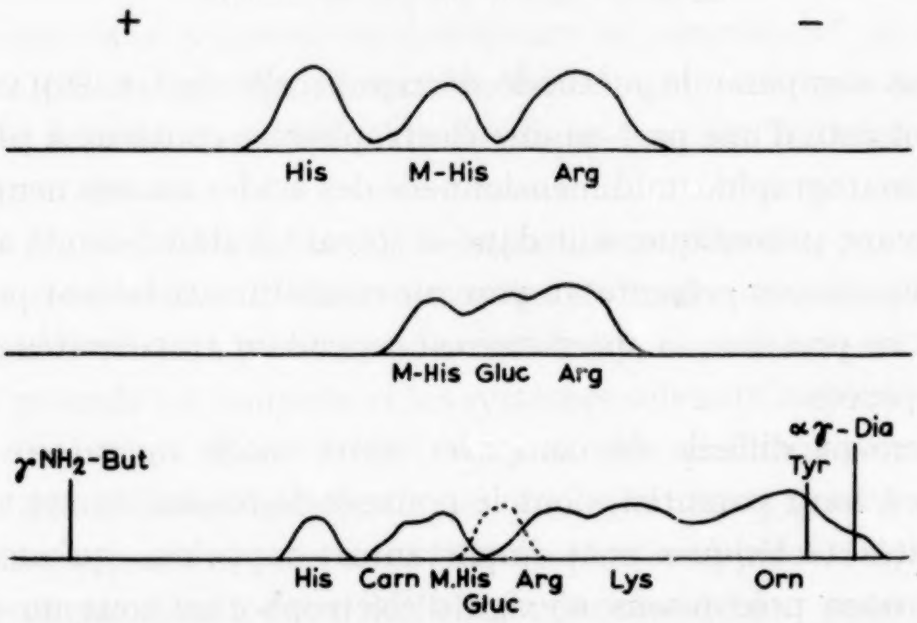

Fig. 13. Electrophorèse des acides aminés basiques en tampon pyridine–acide acétique de pH 6.5 (pyridine: 200 ml; acide acétique: 8 ml; eau: q.s.p. 2000 ml). Appareil d'électrophorèse en V renversé; le papier est placé de façon dissymétrique avec un côté cathodique plus long (30.5 cm) que le côté anodique (26.5 cm). Point de départ du côté cathodique à 28.5 cm du bord de la feuille. Durée de l'électrophorèse: 15 h sous 400 V. $\gamma$-$NH_2$-But = acide $\gamma$-aminobutyrique; His = histidine; M-His = méthylhistidine; Carn = carnosine; Arg = arginine; Gluc = glucosamine; Lys = lysine; Orn = ornithine; Tyr = tyramine; $\alpha,\gamma$-Dia = acide $\alpha,\gamma$-diaminobutyrique.

La présence de ces oligopeptides peut être démontrée, après élution et hydrolyse totale de l'éluat, par la mise en évidence de la β-alanine.

## 4. *Électrophorèse à pH 8.9*

L'électrophorèse en tampon véronal de pH 8.9 ($\mu = 0.1$) permet d'effectuer la séparation de la méthylhistidine et de la carnosine et peut être utilisée pour leur identification à l'aide de témoins latéraux. Cette électrophorèse est très intéressante dans certains cas particuliers, comme celui des extraits musculaires où la carnosine est abondante. Signalons que SCHMIDT ET CUBILES[42] utilisent également l'électrophorèse à pH 9 (solution acétate d'ammonium 0.03 *M* ajustée à pH 9 par de l'ammoniaque 0.1 *N*) pour séparer l'ensemble ansérine + carnosine des autres acides aminés; mais les deux dipeptides restent eux-mêmes confondus.

## 5. *Couplages divers*

Dans certains cas particuliers, l'électrophorèse bidimensionnelle (électrophorèse avec une solution d'ammoniaque 1 *N* dans les deux sens; électrophorèse en première dimension dans l'ammoniaque 1 *N* et dans un tampon borax 0.05 *M*–soude 0.05 *N* de pH 10 en seconde dimension), les couplages "électrophorèse à pH 11.7 ou à pH 6.5 en première dimension et chromatographie dans le solvant butanol–acide acétique–eau en seconde", peuvent quelquefois permettre une séparation intéressante de composés à comportement basique.

### H. DISCUSSION DE LA MÉTHODE

Nous ne pouvons comparer la méthode décrite à celle de GRASSMANN, HANNIG ET PLÖCKL[14], qui consiste d'une part en une électrophorèse continue à pH 3.9 et d'autre part en une chromatographie unidimensionnelle des acides aminés neutres et basiques, soit dans un solvant phénolique, soit dans le solvant butanol–acide acétique–eau. Si le procédé de GRASSMANN présente un pouvoir résolutif satisfaisant pour l'étude d'un hydrolysat total de protéine, sa spécificité est cependant trop limitée pour les milieux biologiques complexes.

Il est également difficile de comparer notre mode opératoire aux méthodes d'électrophorèse à haut potentiel, dont le pouvoir de résolution est très grand, mais dont les difficultés techniques sont importantes; rappelons qu'aucune des étapes successives que nous préconisons n'exige d'électrophorèse sous un potentiel élevé.

La discussion portera donc surtout sur les méthodes d'électrophorèse bidimensionnelle et les autres couplages électrophorèse–chromatographie actuellement décrits.

Les méthodes d'électrophorèse bidimensionnelle les plus connues sont celle de DURRUM[9] (électrophorèse en tampon borate 0.02 *M* de pH 9 sur une bandelette étroite et électrophorèse en milieu acide acétique 0.25 *N* de pH 2.7), celle de MEAD[31] (électrophorèse en tampon phtalate 0.02 *M* de pH 2.5 et électrophorèse en solution ammoniacale de pH 11.6) et celle de WHITEHEAD[49] (électrophorèse en milieu acide acétique 1 *N* en première dimension et en borate de sodium 0.025 *M* de pH 9.2 en seconde dimension). Elles ne présentent aucun avantage sur le mode opératoire que nous proposons et elles ne sont pas sans inconvénients. Dans la méthode de DURRUM, les acides aminés basiques sont perdus; dans celle de MEAD, si la résolution des acides aminés basiques est très satisfaisante, celle des acides à longue chaîne et des composés à caractère acide l'est beaucoup moins; dans celle de WHITEHEAD, la valine et la leucine ne sont pas séparées.

Un certain nombre de couplages électrophorèse–chromatographie ont été publiés, notamment par DURRUM[9], VERHELLE ET MERLEVEDE[46], HONEGGER[21], KICKHÖFEN ET WESTPHAL[25], BLASS, LECOMTE ET POLONOVSKI[5], GERLAXHE ET RENARD[13], MONNIER HUGUET ET GRAS[34], LI et coll.[28], KNAUFF[26], WAGNER[47]. Le principe est toujours sensiblement le même: électrophorèse le plus souvent en milieu acide et chromatographie unidimensionnelle en général dans un solvant butanolique acide.

Citons par exemple les couplages de GERLAXHE ET RENARD[13]: électrophorèse à pH 2.4 et chromatographie dans le solvant butanol–acide formique à 95%–eau (77:15:8); électrophorèse à pH 11.6 et chromatographie dans le solvant butanol–acide formique–eau; chromatographie en phénol tamponné à pH 5.6 et électrophorèse en tampon phosphate de pH 5.6 de force ionique 0.025; celui de KICKHÖFEN ET WESTPHAL[25]: électrophorèse à haut potentiel (70 V/cm) à pH 1.9 en milieu acide acétique 2 *N* et chromatographie dans le mélange pyridine–acide acétique–eau (50:35:15); celui de HONEGGER[21]: électrophorèse en tampon citrate 0.1 *M* de pH 3.8 et chromatographie ascendante dans le solvant *n*-butanol–éthanol–eau–acide acétique (8:4:3:1); celui de KNAUFF[26]: électrophorèse à haut potentiel (80–100 V/cm) dans un tampon acide fumarique–acide acétique de pH 1.9 et chromatographie dans le solvant butanol–acide acétique–eau (4:1:5); celui de LOCKHART ET ABRAHAM[30] et de LI et coll.[28]: électrophorèse à pH 7 dans un tampon collidine–acide acétique–eau et chromatographie dans le solvant butanol–acide acétique–eau ou butanol–acide acétique–pyridine–eau (30:6:20:24).

Dans le cas d'un hydrolysat total, les séparations obtenues par ces procédés sont satisfaisantes et nettement supérieures à celles de la chromatographie bidimensionnelle. Au cours de la mise au point de notre méthode, nous avons été amenés à choisir dans la liste qui précède les tampons et les systèmes solvants les plus efficaces. D'autre part, dans des couplages de cet ordre, il ne semble pas indispensable d'employer l'électrophorèse à haut potentiel: c'est ainsi que les résultats que nous obtenons pour la séparation des composés neutres avec l'électrophorèse à pH 2.4 à potentiel relativement bas sont comparables à ceux de l'électrophorèse à haut potentiel (70 V/cm) à pH 1.9 suivant KICKHÖFEN ET WESTPHAL[25].

Pour les milieux biologiques complexes, les couplages précédents ne permettent qu'une résolution partielle des problèmes posés; le chevauchement des composés neutres et acides, la perte fréquente des composés basiques, constituent souvent des inconvénients majeurs dans les méthodes qui ne comportent qu'une seule étape. C'est pour obtenir le maximum de sélectivité que nous avons proposé un mode opératoire en plusieurs étapes, en retenant notamment un fractionnement de base simple de l'ensemble et en étudiant ensuite plus spécialement la séparation des composés neutres, acides et basiques. Notre combinaison de méthodes présente alors une souplesse plus grande et peut s'adapter à la plupart des cas. Bien que longue, elle n'est jamais fastidieuse et son application aux urines, aux tissus animaux, nous a donné toute satisfaction.

## RÉSUMÉ

Les auteurs décrivent un couplage de méthodes électrophorétiques et chromatographiques classiques applicable à l'étude des acides aminés et de leurs formes combinées dans les milieux biologiques complexes, ainsi qu'à la séparation des oligopeptides. Les étapes essentielles sont les suivantes: fractionnement de base par électrophorèse en tampon volatil de pH 3.9; couplage bidimensionnel comportant une électrophorèse à pH 3.9 et une chromatographie dans le solvant butanol–acide acétique–eau (composés acides et composés faiblement basiques); couplage bidimensionnel comportant une électrophorèse à pH 2.4 et une chromatographie dans le solvant butanol–acide acétique–eau après élution automatique des composés neutres préalablement séparés par le fractionnement de base à pH 3.9; électrophorsèe à pH

11.7 et à pH 6.5 après élution automatique des composés basiques préalablement séparés par le fractionnement de base à pH 3.9.

Le procédé reste commode et d'une exécution relativement facile; il ne nécessite qu'un appareillage simple et peu coûteux.

## SUMMARY

The authors describe a combination of electrophoretic and classical chromatographic methods, which can be used for investigating free amino-acids or amino-acid compounds in complex biological material, as well as for separating oligopeptides. The essential steps in this procedure are the following: preliminary fractionation by electrophoresis in a volatile buffer of pH 3.9; a two-dimensional combination of electrophoresis at pH 3.9 and chromatography in the solvent butanol–acetic acid–water (acid and weakly basic compounds); a two-dimensional combination of electrophoresis at pH 2.4 and chromatography in the solvent butanol–acetic acid–water, after automatic elution of the neutral compounds separated by the preliminary fractionation at pH 3.9; electrophoresis at pH 11.7 and at pH 6.5 after automatic elution of the basic compounds separated by the preliminary fractionation at pH 3.9.

The method is convenient and comparatively easy to carry out; only simple and inexpensive apparatus is required.

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# CONSIDÉRATIONS GÉNÉRALES SUR LA CHROMATOGRAPHIE D'IONS INORGANIQUES SUR CELLULOSE

J. FOUARGE* ET G. DUYCKAERTS
*Laboratoire de Chimie analytique, Université de Liège (Belgique)*

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## INTRODUCTION

Il est fréquemment admis et à plusieurs reprises, il a été prouvé[1-3, 22] que la séparation chromatographique sur cellulose est basée sur le partage entre la phase mobile éluante et une phase aqueuse immobilisée dans la cellulose.

La théorie de la chromatographie de partition développée par MARTIN ET SYNGE[1] permet alors de prévoir raisonnablement les positions relatives des différents pics d'élution moyennant la connaissance des coefficients de partage de chaque substance.

Si ce mécanisme de partage se comprend et se vérifie facilement lorsque l'éluant est peu miscible à l'eau, l'obtention de séparations satisfaisantes à l'aide de solvants entièrement miscibles à l'eau, tel que le méthanol, semble difficile à interpréter de façon identique; de plus, il est possible d'obtenir avec HCl 12 *N* pur, sans aucune addition de solvants organiques, des séparations de certains cations.

Nous allons montrer que le comportement chromatographique des alcalins, des alcalino-terreux et des terres rares qui, en milieu chlorhydrique, ne forment pas de complexes stables, ne peut guère, dans certaines conditions, s'interpréter que par un mécanisme d'adsorption.

## ELUTIONS PAR LE PHÉNOL ÉQUILIBRÉ AVEC HCl 2 *N*

Parmi les ions considérés, seuls les alcalins Cs, Rb, K migrent avec une vitesse appréciable[4, 5]. Sur bande de papier Whatman No. 1, les $R_F$ valent approximativement: Cs (0.52); Rb (0.27); K (0.19).

Remarquons d'abord que l'ordre d'élution est inverse de celui obtenu sur des résines échangeuses, ou sur cellulose avec des solvants non phénoliques, notamment les alcools.

Nous avons également noté sur les enregistrements[5, 6] que le pic de $^{137}$Cs "carrier free" est relativement étroit, quasi symétrique, et en forme de cloche comme le prévoit la théorie; avec des doses pondérables de césium, le pic tend à s'étaler un peu, généralement du côté de l'orgine.

Nous avons la conviction que, dans le cas envisagé, la séparation résulte de

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[*] Chercheur agréé à l'Institut Interuniversitaire des Sciences Nucléaires.

partages du type liquide–liquide entre la phase phénolique mobile et la phase aqueuse immobilisée sur la cellulose; en effet, en l'absence de support cellulosique, on observe un partage de ces alcalins entre les deux phases en question et les coefficients de distribution ($K_d$ org./aq.) que nous avons mesurés (Tableau I), concordent raisonnablement avec ceux calculés sur la base des positions relatives des pics d'élution, en appliquant les relations classiques:

$$K_d(\mathrm{B}) = K_d(\mathrm{A}) \times \frac{1/R_F\,(\mathrm{A}) - 1}{1/R_F\,(\mathrm{B}) - 1} \text{ (bandes)}$$

ou

$$K_d(\mathrm{B}) = K_d(\mathrm{A}) \times \frac{V_m\,(\mathrm{A}) - V_L}{V_m\,(\mathrm{B}) - V_L} \text{ (colonnes)}$$

TABLEAU I

| *Élément* | $R_F$ | $K_d$ *mesuré* | $K_d$ *calculé (bandes)* | $V_m/V_L$[11] | $K_d$ *calculé (colonne)* |
|---|---|---|---|---|---|
| Cs | 0.52 | $0.26_3 \pm 3\%$ | — | 1.74 | — |
| Rb | 0.27 | 0.086 | 0.090 | 3.30 | 0.085 |
| K | 0.19 | 0.047 | 0.057 | 4.82 | 0.051 |

Signalons que nous avons étudié de façon plus approfondie le partage de CsCl entre une phase aqueuse chlorhydrique et la phase phénolique en équilibre, en nous aidant de $^{137}$Cs "carrier free" comme indicateur; nous avons notamment vérifié que $K_d$ demeure constant: $K_d = 0.26$ ($\pm$ 3%) à 22° dans un domaine de concentration en CsCl de $10^{-9}$ et $10^{-1}$ $M$ quand la phase aqueuse est 2 $N$ en HCl[6].

Ce sont là tous arguments permettant de conclure qu'il s'agit bien d'une chromatographie de partition entre phases liquides.

## CHROMATOGRAPHIE SUR CELLULOSE AVEC DES ÉLUANTS À BASE D'ALCOOLS

### *A. Valeur des $R_F$, ordre d'élution, variation du $R_F$ avec le teneur en eau de l'éluant*

En utilisant un solvant organique pur, des $R_F$ utiles ($> 0.2$) ne s'obtiennent pratiquement qu'avec le méthanol[7-10]; si l'on désire utiliser d'autres alcools, ceux-ci doivent être additionnés d'une certaine proportion d'eau (5 à 20% en volume).

D'une manière générale, on constate que l'élution des ions considérés s'accélère lorsque la teneur en eau de l'éluant augmente[2, 7, 8] et pour un certain nombre, KERTES[11] a observé une augmentation plus ou moins linéaire du $R_F$ avec la constante diélectrique de l'éluant additionné de quantités croissantes d'eau.

Il existe souvent un parallélisme entre le $R_F$ et la solubilité dans l'éluant; dans le cas que nous considérons, ce ne doit pas être le facteur déterminant: en effet, CsCl est beaucoup plus soluble dans le méthanol que KCl[12].

Dans chaque famille d'éléments, l'ordre d'élution sur cellulose est, tout comme sur résines, celui des rayons ioniques hydratés décroissants.

Il est d'ailleurs à signaler que le méthanol solvate aisément les ions alcalins et alcalino-terreux, principalement les premiers termes; on connaît en effet des composés tels que: $LiCl(CH_3OH)_3$; $MgCl_2(CH_3OH)_6$; $CaCl_2(CH_3OH)_4$[13].

Il semble donc que dans ce cas le rayon ionique hydraté (ou solvaté) constitute en fait le principal facteur déterminant les valeurs relatives de $R_F$; nous pouvons encore noter à l'appui de cette thèse que les vitesses de Rb et Cs sont pratiquement identiques dans tous les mélanges éluants organiques essayés, à l'exception des phénols.

Effectivement, dans l'eau, le méthanol ou l'éthanol, les valeurs relatives* des "rayons ioniques solvatés" de Rb et Cs sont identiques[14] (voit Tableau II).

TABLEAU II

| *Solvant* | *Rayon* | *Li⁺* | *Na⁺* | *K⁺* | *Rb⁺* | *Cs⁺* |
|---|---|---|---|---|---|---|
| | $R_{crist}$ Å | 0.60 | 0.95 | 1.33 | 1.48 | 1.69 |
| Eau | $R_{solv.}$ Å | 2.84 | 2.19 | 1.50 | *1.41* | *1.42* |
| Méthanol | $R_{solv.}$ Å | 4.53 | 3.94 | 3.37 | *3.14* | *3.14* |
| Éthanol | $R_{solv.}$ Å | 6.05 | 4.83 | 4.10 | *3.82* | *3.82* |

Il nous paraît vraisemblable d'admettre qu'en l'absence de complexant, au sens classique du terme, les "moteurs" de la migration sont les molécules solvatantes elles-mêmes et à ce point de vue, l'eau occupe une place de choix; plus il y aura d'eau disponible dans l'éluant, plus les ions seront hydratés et par suite, moins ils seront retenus sur la cellulose. L'expérience montre effectivement que le $R_F$ augmente avec la proportion d'eau ajoutée à l'éluant.

Sur la base de cette hypothèse, on peut comprendre qualitativement l'action freinante des acides non complexants (voir paragraphe B) ajoutés à l'éluant à l'état pur ou concentré: ils s'emparent tout simplement d'une fraction croissante de l'eau libre qui, de ce fait, n'est plus disponible pour l'hydratation, la désorption et le transport des ions. Seuls des ions à énergie d'hydratation élevée ($Li^+$, $Mg^{++}$) pourront entrer sérieusement en compétition avec les ions $H^+$.[8]

### *B. Variation du $R_F$ avec la proportion de HCl ajoutée au méthanol*

L'addition au méthanol de quantités de HCl 12 *N* (inférieure à 20%) diminue progressivement le $R_F$; le fait a été observé pour les alcalino-terreux élués sur bande de papier ou sur colonne de cellulose et pour les alcalins sur bande de papier[7,8].

Lorsque la proportion de solvant organique tombe en dessous d'une certaine limite, variable avec l'élément, on observe une recroissance des $R_F$ due à l'effet prépondérant de l'eau apportée par l'acide. Dans le cas des alcalins élués sur colonne de cellulose, l'effet se marque déjà pour des teneurs de quelques pourcents[6].

Avec des éluants moyennement riches en HCl 12 *N* (50%) additionnés de méthanol et de diverses cétones, on observe déjà des inversions dans l'ordre habituel d'élution des alcalins[15]; on a: $R_F$ [Cs > Rb > K > Na]; Li migre près de Cs.

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* Ces chiffres n'ont qu'une valeur relative puisque, comme l'auteur le fait remarquer, les "rayons solvatés" de Rb et Cs en solution dans l'eau sont trouvés plus petits que les rayons cristallographiques.

A la limite, avec un éluant aqueux exempt de solvant organique, on obtient un $R_F$ voisin de 1; si l'on élue avec HCl de concentration allant de *N* à 6 *N*, tous les alcalins, les alcalino-terreux et les terres rares migrent pratiquement avec le front du liquide; on observe toutefois un léger retard de Sr élué par HCl 6 *N*.

Nous allons voir qu'en utilisant HCl 12 *N* seul comme éluant, on obtient de nouveau, particulièrement pour les alcalino-terreux, des $R_F$ nettement inférieurs à 1.

## ÉLUTIONS SUR PAPIER AVEC HCl 12 *N*

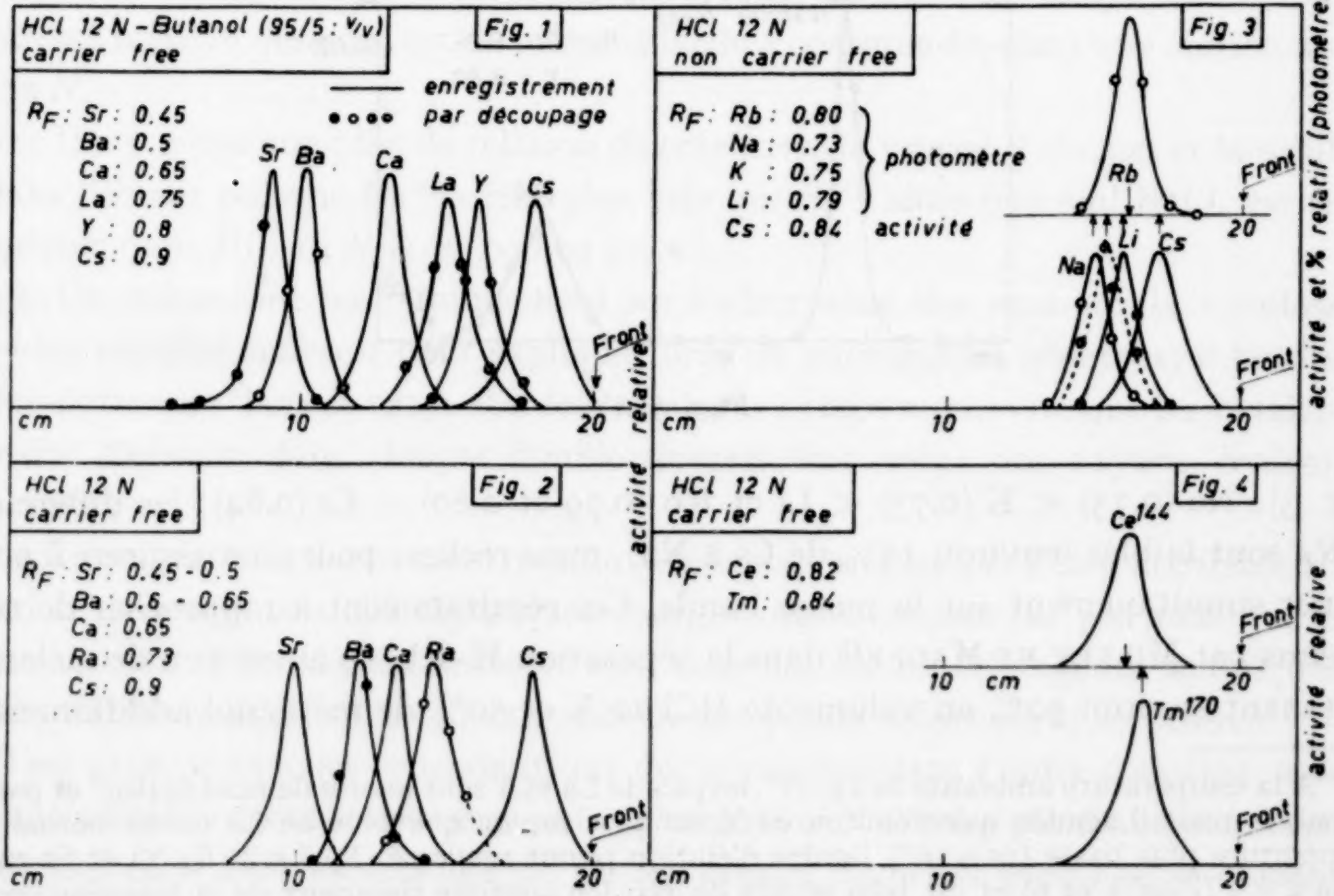
Nous avions signalé dans une publication antérieure[7] que $^{140}$Ba et $^{90}$Sr en quantités impondérables migrent ensemble ($R_F = 0.5$) lorsqu'on utilise un éluant très riche en HCl 12 *N* proposé dans la littérature (HCl 12 *N*-butanol 95:5 en volume).

La séparation forcément partielle obtenue par les auteurs, qui travaillaient avec des quantités pondérables de substances, résultait uniquement de la très faible solubilité de $BaCl_2$ dans l'éluant.

Nous avons remarqué que dans les mêmes conditions $^{90}$Y et $^{140}$La s'éluent respectivement à $R_F = 0.8$ et 0.75, ils sont inséparables entr'eux mais tous bien séparés de leurs parents radioactifs $^{90}$Sr et $^{140}$Ba. $^{45}$Ca migre avec $R_F = 0.65$ (Fig. 1).

Nous nous sommes demandé si dans un éluant aussi riche en acide chlorhydrique, la présence de butanol était bien indispensable pour obtenir une élution retardée par rapport au front du liquide.

Cela nous a amené à essayer l'élution avec HCl 12 *N* seul. Nous avons opéré sur bandes de papier Whatman No. 1 (1 ou 2.5 × 20 cm utile), en élution descendante



Figs. 1–4.

comme d'habitude[7]. Il est à noter que le papier devient fragile et difficile à manipuler, même après séchage.

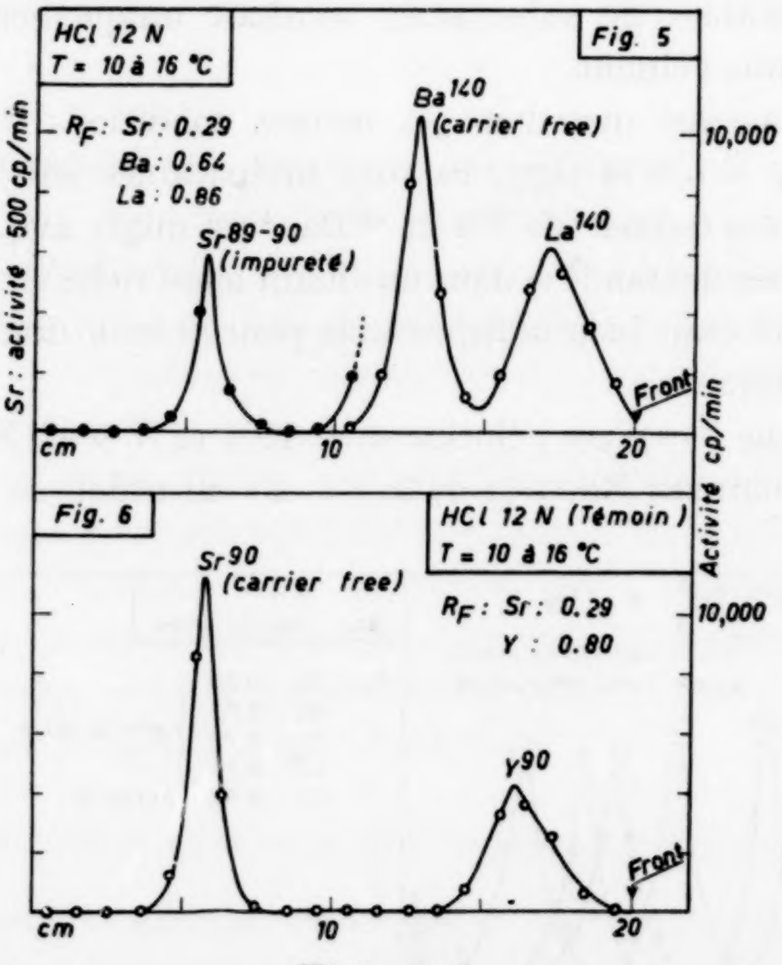
Avec HCl 12 *N*, et des doses traceurs nous avons obtenu les $R_F$ suivants: (Figs. 2 et 4).

| | | | | | |
|---|---|---|---|---|---|
| $Cs^+$ | 0.85–0.90 | $Ca^{+2}$ | 0.65 | $Y^{+3}$ | 0.75–0.9 |
| | | $Sr^{+2}$ | 0.45–0.50 | $La^{+3}$ | 0.75–0.9 |
| | | $Ba^{+2}$ | 0.6–0.65 | $Ce^{+3}$ | 0.82 |
| | | $Ra^{+2}$ | 0.73 | $Tm^{+3}$ | 0.84 |

Certes, d'une expérience à l'autre, on peut obtenir des différences de $R_F$ (10%) mais les valeurs relatives restent les mêmes*.

Nous avons de même essayé l'élution de quantités pondérables de Li, Na, K, Rb et Cs; les quatre premiers étant détectés au spectrophotomètre de flamme et Cs par l'activité de $^{137}Cs$ ajouté comme traceur (Fig. 3).

Dans un essai particulier, les $R_F$ obtenus s'échelonnent dans l'ordre suivant (Fig. 3): Na (0.73) < K (0.75) < Li et Rb (0.79 et 0.80) < Cs (0.84); les différences de $R_F$ sont faibles (environ 15% de Cs à Na), mais réelles; pour s'en assurer, il suffit d'éluer simultanément sur la même bande. Ces résultats sont à rapprocher de ceux obtenus par MILLER ET MAGEE[15] dans la séparation K–Rb–Cs au moyen de mélanges contenant environ 50% en volume de HCl 12 *N* et 50% de méthanol additionnés de



Figs. 5 et 6.

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* A la température ambiante de 24–28°, les pics de La et Y sont anormalement étalés[21] et parfois dédoublés mais il semble que l'élution de Y est plus rapide que celle de La (ordre normal). A température plus basse (10 à 16°), l'ordre d'élution paraît renversé ($R_F$ La > $R_F$ Y) et Sr recule jusqu'à 0.3 (Figs. 5 et 6) et est bien séparé de Ba. Un contrôle rigoureux de la température, de préférence en dessous de la normale, est nécessaire pour obtenir des résultats reproductibles.

diverses cétones; ils obtiennent des $R_F$ croissants dans l'ordre: Na < K < Rb < Cs et Li.

En conclusion, mis à part Li et Ca, on observe donc une inversion dans l'ordre d'élution par rapport à ce qui avait été trouvé pour le méthanol.

## DISCUSSION

Puisque l'élution avec l'eau acidulée par HCl $N$[6, 16] donne des $R_F$ voisins de 1, quel est le frein qui, dans l'élution avec HCl 12 $N$, provoque une rétention qui est particulièrement importante pour les alcalino-terreux? Par ailleurs, comment expliquer l'ordre d'élution dans chaque famille?

*Alcalins*: rétention moyenne dans l'ordre d'importance croissante: Cs, Li et Rb, K, Na.

*Alcalino-terreux*: rétention importante spécialement pour Sr; l'ordre d'importance croissant est: Ra, Ca, et Ba, Sr.

*Terres rares*: Légère rétention; ordre d'importance croissant apparemment inchangé par rapport à l'ordre habituel d'élution sur résines ou cellulose, caractérisé par: Tm < Ce.

A première vue, une rétention aussi importante peut paraître surprenante, d'autant plus qu'elle se fait sentir de manière spéciale pour les alcalino-terreux; le mécanisme paraît être différent de ceux habituellement invoqués, en effet:

(a) Un mécanisme basé sur l'échange d'ions avec la cellulose seulement est hors de question, car ses rares groupements –COOH ont un caractère acide faible et dans ce cas d'ailleurs, les trivalents devraient s'éluer en dernier lieu[24].

(b) Un partage du type liquide–liquide auquel on pourrait songer étant donné l'étroitesse relative des pics, est de même difficile à comprendre dans une élution avec HCl 12 $N$.

(c) Il n'y a pas non plus de relation directe entre la vitesse d'élution et la solubilité dans l'éluant puisque $Ba^{+2}$ s'élue plus vite que $Sr^{+2}$ alors que seul $BaCl_2$ est très peu soluble dans HCl 12 $N$ (1/20,000 en poids).

(d) Un mécanisme basé uniquement sur l'adsorption des ions sur la cellulose[23] ne semble pas être suffisant pour expliquer tous les phénomènes observés; il faudrait en effet s'attendre dans ce cas à voir les trivalents s'éluer moins vite que les bivalents et l'ordre d'élution dans chaque famille devrait être celui des rayons hydratés décroissants.

(e) La migration relativement rapide des terres rares ne peut être attribuée à la formation de complexes chlorhydriques chargés négativement car pas plus que les alcalino-terreux et les alcalins, elles ne sont retenues sur les échangeurs anioniques[4]. On a cependant mis en évidence l'existence de complexes chlorhydriques positifs[17].

Il est utile de signaler cependant que des inversions dans l'ordre d'élution, assez semblables à celles décrites ci-dessus, ont également été observées par d'autres auteurs, précisément dans l'élution sur résines de ces mêmes éléments avec l'acide chlorhydrique concentré (> 9 $N$). DIAMOND et coll.[18, 19] mesurant les coefficients de partage de toute une série d'éléments (Na, Cs, alcalino-terreux, terres rares et transuraniens) entre l'acide chlorhydrique de concentration variable et une résine cationique, constatent que:

(a) Aux concentrations en acide chlorhydrique inférieures à 5-6 *N*, les coefficients de distribution ($K_d$) décroissent de manière monotone avec l'augmentation de la concentration de l'acide (effets de masse). Les courbes sont à peu près parallèles pour les éléments d'une même famille.

(b) Aux concentrations supérieures à 7 *N*, les $K_d$ d'un certain nombre de ces éléments notamment ceux de Na, Cs, Sr et, dans une moindre mesure, ceux des terres rares se remettent à croître rapidement d'autant plus vite que le rayon de l'ion hydraté est grand; il en résulte au delà de 12 *N* un renversement complet de l'ordre normal d'élution des alcalino-terreux et des alcalins représentée par Cs et Na. L'ordre d'élution des terres rares reste par contre inchangé.

Comme le postulent les auteurs, un des principaux facteurs responsables de ces phénomènes est, à n'en pas douter, la déshydratation progressive des ions par l'acide concentré. Il faut se rappeler, en effet, que dans HCl 12 *N*, il y a seulement 3.6 môles d'eau pour 1 môle d'acide et la tension de vapeur indique que HCl 12 *N* est loin d'être hydraté à saturation. Dans ces conditions les ions étrangers, les polyvalents en premier lieu, vont entrer en compétition pour l'eau avec les ions $H^+$ et de ce fait perdront une partie, voire la totalité, de l'eau d'hydratation qu'ils possèdent normalement en solution. Comme la fixation sur la résine est de nature essentiellement électrostatique, elle augmente avec la charge et elle diminue avec le rayon ionique effectif en solution; on comprend alors que la déshydratation des ions qui se marque au delà de [HCl] = 6 à 7 *N*, puisse provoquer une augmentation du $K_d$ et finalement un renversement dans les ordres d'élution qui deviennent: Ra, Ba, Sr, Ca et Cs, Rb, K, Na, Li c'est à dire l'ordre des rayons ioniques cristallins décroissants. On sait en effet que dans une même famille, les rayons ioniques cristallins croissent avec le nombre atomique tandis que ceux des ions hydratés décroissent.

On comprend mieux dès lors que, si c'est l'adsorption qui est le facteur prédominant sur cellulose, on puisse observer des phénomènes similaires de rétention allant jusqu'à un renversement de l'ordre d'élution des ions lorsque ceux-ci sont suffisamment déshydratés.

Le fait que sur bande de papier, $Li^+$ et $Ca^{+2}$ occupent des positions spéciales est vraisemblablement dû à la petite dimension et à l'énergie d'hydratation élevée de ces ions qui ne sont pas encore complètement déshydratés dans nos conditions de travail. Ce sont précisément des cations qui peuvent remplacer les ions $H^+$ comme relargants dans certaines extractions de sels par des solvants organiques[20].

## REMARQUE SUR LA LARGEUR DES PICS D'ÉLUTION

Dans l'élution du $^{137}$Cs par le phénol[5], le pic est relativement étroit; on peut calculer, d'après la théorie de MARTIN ET SYNGE, que la "hauteur équivalente à un plateau théorique" est, sans même tenir compte de la dimension non négligeable de la tache d'origine, de l'ordre de 0.01 à 0.05 cm; une colonne de 8 cm de haut est bien suffisante pour obtenir une excellente séparation Cs–Rb, malgré un rapport des vitesses d'élution (Cs/Rb) légèrement inférieur à 2.[5]

Par contre, les pics obtenus avec les éluants à base de méthanol pauvre en eau sont beaucoup plus larges; il faut une colonne de 40 cm de long et un rapport des $R_F$ (Sr/Ba) de 3 au moins pour obtenir une séparation Ba–Sr quantitative.

Sur bandes de papier, les pics de $^{140}$Ba ($R_F = 0.07$) et $^{90}$Sr ($R_F = 0.25$), élués par le mélange méthanol–HCl 12 *N* (100:5, v/v)[7], bien que symétriques et en forme de cloches, sont beaucoup plus larges que celui de $^{137}$Cs élué au phénol malgré le $R_F$ plus élevé de ce dernier (0.5). La hauteur équivalente à un plateau théorique est de l'ordre de 0.5 cm cette fois, soit de 10 à 50 fois celle de l'élution au phénol.

Il y a là encore une indication, pour les élutions au méthanol, d'un mécanisme différent de celui d'un partage liquide–liquide.

Nous ajouterons que l'addition de quantités croissantes d'eau aux éluants à base de méthanol non seulement accélère l'élution mais souvent rétrécit les pics, ce qui ne s'expliquerait pas par un mécanisme de chromatographie de partition.

On doit admettre qu'il y a une augmentation du nombre de plateaux avec la teneur en eau de l'éluant et, comme celle-ci s'accompagne d'une augmentation de l'hydratation de la cellulose, il semble logique d'admettre que la surface nécessaire à la formation de l'équivalent d'un plateau se développe en profondeur, à l'intérieur des fibres dont une part de plus en plus grande participe à la formation de la phase fixe.

## CONCLUSIONS

Bien que pour la séparation des alcalins sur cellulose par le phénol, le processus soit incontestablement du type partage liquide–liquide, il nous semble que les élutions par le méthanol (éluant entièrement miscible à l'eau) sont à rattacher plutôt à la chromatographie d'adorption, avec cette particularité que le siège du phénomène ne semble pas limité à la surface des fibres de cellulose mais qu'il s'étend à une profondeur variable avec la teneur en eau de l'éluant. Il est probable qu'il en est de même avec les autres alcools entièrement miscibles à l'eau.

Pour les alcalins, les alcalino-terreux et les terres rares examinés, le rayon de l'ion solvaté, fixé par la teneur en eau ou en méthanol, est un facteur important.

Lorsqu'on élue par HCl 12 *N* exempt de tout solvant organique, on doit admettre l'influence de l'adsorption sur la cellulose et celui du rayon ionique solvaté pour comprendre les rétentions et les inversions de l'ordre normal d'élution de la plupart des ions considérés.

## REMERCIEMENTS

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## RÉSUMÉ

Certains résultats caractéristiques obtenus pour des séparations inorganiques sur cellulose (alcalins, alcalino-terreux et terres rares) sont discutés du point de vue du mécanisme.

Alors que l'élution des alcalins sur cellulose au moyen du phénol équilibré avec HCl 2 *N* s'avère incontestablement être du type partition entre deux phases liquides, les séparations au moyen de solvants à base de méthanol doivent plutôt être rattachés à la chromatographie d'adsorption.

Dans ce dernier cas, il apparaît que ce sont le plus souvent les molécules "d'eau libre" de l'éluant qui jouent le rôle d'agent moteur. Cette hypothèse permet de comprendre qualitativement l'action rétensive due à l'addition d'acides à l'éluant: les ions $H^+$ fixent l'eau libre.

De plus, pour l'élution par HCl 12 *N* pur, on observe des inversions dans ce qui est l'ordre habituel des élutions sur cellulose: celui des rayons ioniques hydratés décroissants.

Les rétentions et inversions avec HCl 12 *N* sont certainement dues à la déshydratation partielle des cations par les ions $H^+$ de l'acide.

Enfin, la largeur relativement grande des pics obtenus avec les éluants à base de méthanol est une indication supplémentaire en faveur d'un processus d'adsorption.

## SUMMARY

Some characteristic results of inorganic separations (alkalis, alkaline earths and rare earths) on cellulose are reviewed from the point of view of the elution mechanism.

It is shown that elution of alkalis adsorbed on cellulose by phenol equilibrated with 2 *N* HCl is undoubtedly due to partition between two liquid phases, whereas elution with methanolic eluants must to be considered as a process of adsorption chromatography.

The authors consider, that in the latter case the "free water" molecules of the eluant must be regarded as the migration "motor". This hypothesis provides an explanation for the retarding action of acids: the $H^+$ ions bind "free water" molecules.

Furthermore, on elution of the same cations adsorbed on cellulose with pure 12 *N* HCl an inversion of the normal order of elution (*i.e.* in the order of decreasing radius of the hydrated ions), is observed.

The inversion and the retarding action are undoubtedly due to the cation being dehydrated by the $H^+$ ions of the acid (increased adsorbability due to a smaller cationic radius).

Finally, the comparatively wide peaks obtained with methanolic eluants is a further indication that an adsorption process is involved.

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# QUELQUES SÉPARATIONS DE RADIOISOTOPES AU MOYEN DE L'ACIDE ÉTHYLÈNEDIAMINETETRAACÉTIQUE

G. DUYCKAERTS ET R. LEJEUNE
*Laboratoire de Chimie analytique, Université de Liège (Belgique)*

(Reçu le 26 mars 1959)

Dans le seul domaine de la chimie analytique, l'acide éthylènediaminetétraacétique (EDTA) a trouvé des applications multiples[1] d'un intérêt considérable; en chromatographie, son emploi a été également souvent proposé: il forme en effet des complexes de chelation avec la plupart des cations et les différences de stabilité permettent aisément d'obtenir des séparations sur échangeurs cationique ou anionique; très souvent, les différences de stabilité sont telles qu'il suffit de choisir un pH où un groupe de cations est entièrement complexé et par conséquent fixé sur résine anionique et non fixé sur résine cationique tandis que l'autre groupe n'est pas complexé et se sépare complètement par passage sur la résine; c'est le cas de la séparation de cations de charges différentes[2–7].

Lorsqu'il s'agit de séparer des cations de même charge, par exemple ceux d'une même famille, les résultats sont aussi satisfaisants: il suffit de citer le cas de la séparation des terres rares ou des transuraniens: même dans ces familles, les complexes possèdent des stabilités suffisamment différentes que pour obtenir des séparations très bonnes[8–11]; la méthode a été exploitée à la fois à l'échelle analytique et à l'échelle industrielle.

Dans notre laboratoire, différents chercheurs ont eu l'occasion d'étudier les possibilités d'utilisation de l'EDTA en chromatographie sur Dowex 50 en utilisant soit la technique classique par élution à l'EDTA amené au pH convenable par neutralisation à l'ammoniac[12] soit la technique d'élution avec un complexe d'EDTA; dans ce dernier cas, la résine est mise sous forme du même métal[13].

Dans cette note, nous nous proposons de donner quelques résultats obtenus dans la préparation de radioisotopes carrier-free.

## *Détails expérimentaux*

Tous les essais ont été faits sur Dowex 50 calibré entre 35 et 75 $\mu$ par sédimentation; la résine est sous forme ammonique et possède une capacité d'échange de 5.2 meq. g/g de résine sèche.

L'élution est effectuée au moyen d'une solution $10^{-2}$ *M* en EDTA amenée au pH convenable par neutralisation à l'ammoniac. La colonne a une section de 0.78 cm$^2$; le poids de résine sèche est de l'ordre de 15 g (volume libre: environ 10 ml). Les séparations se font à température ambiante. Les mesures d'activité sont faites au moyen d'un tube G.M. Tracerlab T.G.C.2 et d'une installation de comptage Nuclear.

## *Séparation $^{140}$Ba–$^{90}$Sr et de leurs produits de filiation*

Dans une publication antérieure[12], nous avions déterminé les coefficients de partage du Ba et Sr entre le Dowex 50 et une solution d'EDTA à différents pH et montré que les valeurs étaient en parfait accord avec celles calculables à partir des p$K$ de l'acide et des constantes de stabilité des complexes. Cela nous a incité à calculer les courbes de $K_d$ en fonction du pH pour les différents cations, de façon à pouvoir connaître à priori, de façon assez précise, le pH optimum pour une séparation donnée.

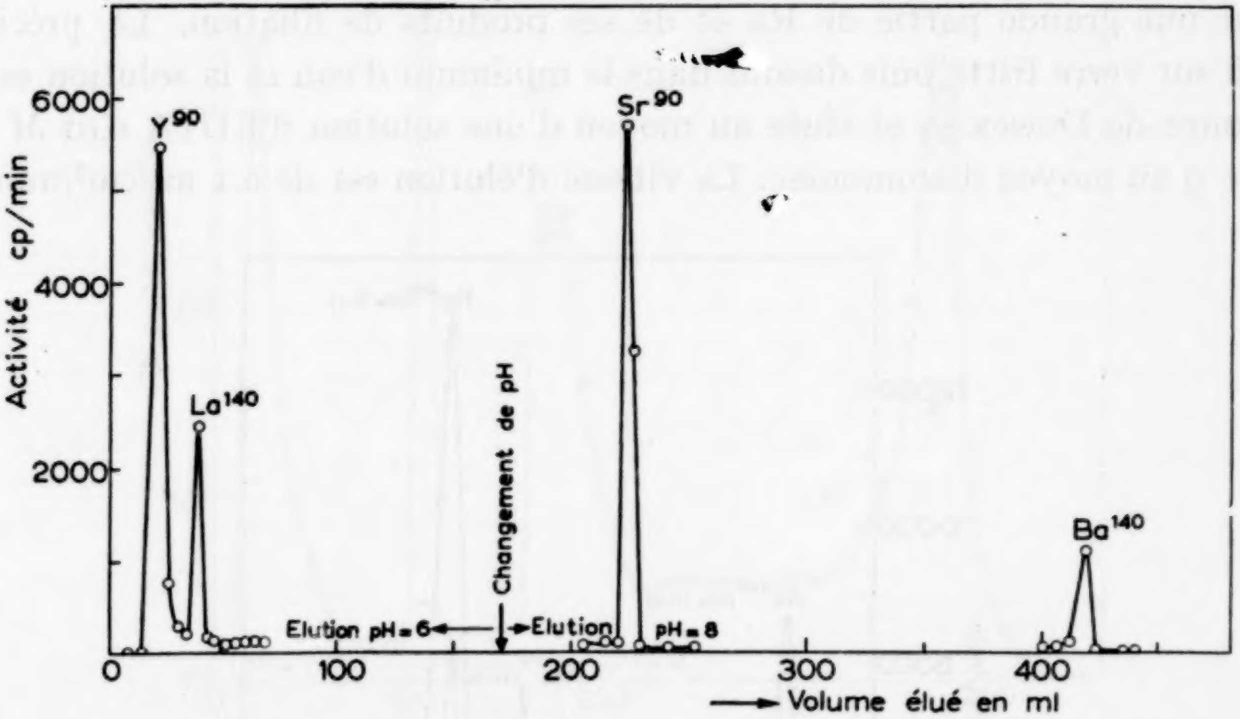

Fig. 1. Séparation chromatographique $^{140}$Ba–$^{90}$Sr de fission et de leurs produits de filiation par l'EDTA.

La Fig. 1 donne un exemple de séparation $^{140}$Ba–$^{90}$Sr de fission sans entraîneur et de leurs produits de filiation respectifs $^{140}$La et $^{90}$Y.

La colonne sous forme ammonique est d'abord lavée au moyen du mélange éluant à pH = 6 jusqu'à équilibre; puis rincée avec 50 ml d'eau désionisée; la solution contenant Ba–Sr dans un minimum de volume à pH 3.7–3.8 est versée sur la colonne puis éluée à pH = 6; lorsque les deux premiers pics sont passés, on amène le pH de l'éluant vers 8.

Des essais en fonction du pH ont montré que les pH 6 et 8.5 fournissaient des séparations satisfaisantes dans des temps raisonnables.

## *Préparation de $^{228}$Ra*

En vue d'essayer une séparation plus complète de la famille des alcalino-terreux, nous avons eu à préparer du radium. Nous nous sommes adressés à la famille du $^{232}$Th qui contient $^{228}$Ra (MsTh$_1$) et $^{224}$Ra (ThX).

$$^{232}\mathrm{Th} \xrightarrow[1.39\cdot10^{10}\mathrm{a}]{\alpha} {}^{228}\mathrm{Ra}\ (\mathrm{MsTh_1}) \xrightarrow[6.7\mathrm{a}]{\beta} {}^{228}\mathrm{Ac}\ (\mathrm{MsTh_2}) \xrightarrow[6.13\mathrm{h}]{\beta}$$

$$^{228}\mathrm{Th(RdTh)} \xrightarrow[1.90\mathrm{a}]{\alpha} {}^{224}\mathrm{Ra(ThX)} \xrightarrow[3.64\mathrm{j}]{\alpha} {}^{220}\mathrm{Rn} \xrightarrow[54.5\mathrm{s}]{\alpha} {}^{216}\mathrm{Po(ThA)} \xrightarrow[0.158\mathrm{s}]{\alpha}$$

$$^{212}\mathrm{Pb(ThB)} \xrightarrow[10.6\mathrm{h}]{\beta} {}^{212}\mathrm{Bi(ThC)} \xrightarrow[60.5\mathrm{m}]{\alpha} {}^{208}\mathrm{Tl(ThC'')} \xrightarrow[3.1\mathrm{m}]{\beta} {}^{208}\mathrm{Pb}$$

Pour séparer $^{228}$Ra et $^{224}$Ra présents dans le nitrate de thorium, nous avons ajouté à la solution contenant 3 g de $Th(NO_3)_4$ dans quelques ml d'eau acidulée par HCl pour chasser $HNO_3$, 0.05 g de $BaCl_2$ dissous dans l'eau; le chlorure de baryum est précipité par addition de 5 à 6 fois son volume de HCl 12 *N*. Le précipité de $BaCl_2\cdot 2H_2O$ entraîne une grande partie de Ra et de ses produits de filiation. Le précipité est recueilli sur verre fritté puis dissous dans le minimum d'eau et la solution est versée sur colonne de Dowex 50 et éluée au moyen d'une solution d'EDTA 0.01 *M* amenée à pH = 9 au moyen d'ammoniac. La vitesse d'élution est de 0.1 ml/cm²/min.

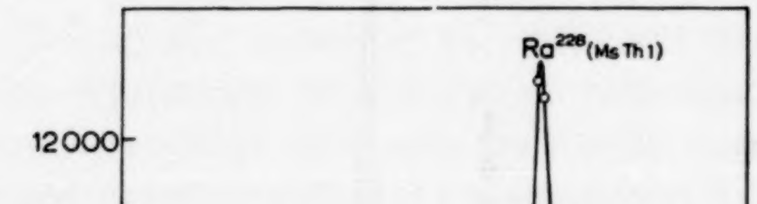

Fig. 2. Séparation chromatographique à l'EDTA de produits de filiation du $^{232}$Th.

La Fig. 2 montre le chromatogramme obtenu, mesuré directement après l'élution; la courbe en traits interrompus a été obtenue après 8 jours; tous les pics, à l'exception de celui du radium 228 ont disparu. La mesure des décroissances a permis de faire les attributions indiquées sur la figure. On voit de nouveau que les éléments s'éluent dans l'ordre des charges décroissantes. Le fond continu qui se marque entre le pic de

Ac et celui de Ra directement au sortir de la colonne provient des produits de filiation qui se forment à partir du $^{224}$Ra au cours de son parcours dans la colonne; il ne s'agit donc pas d'un manque de pouvoir de séparation de la colonne. Après 8 jours, toutes les vies courtes ont disparu et le pic restant est dû au $^{228}$Ra.

### *Séparation Ca–Sr–Ba–Ra*

Cette séparation a été réalisée sur une solution contenant des quantités traceurs de $^{45}$Ca, $^{90}$Sr, $^{140}$Ba et $^{228}$Ra. La solution est amenée à pH = 3 sur la colonne, éluée au complexon à pH = 7.4 d'abord, puis à pH = 9 après le strontium.

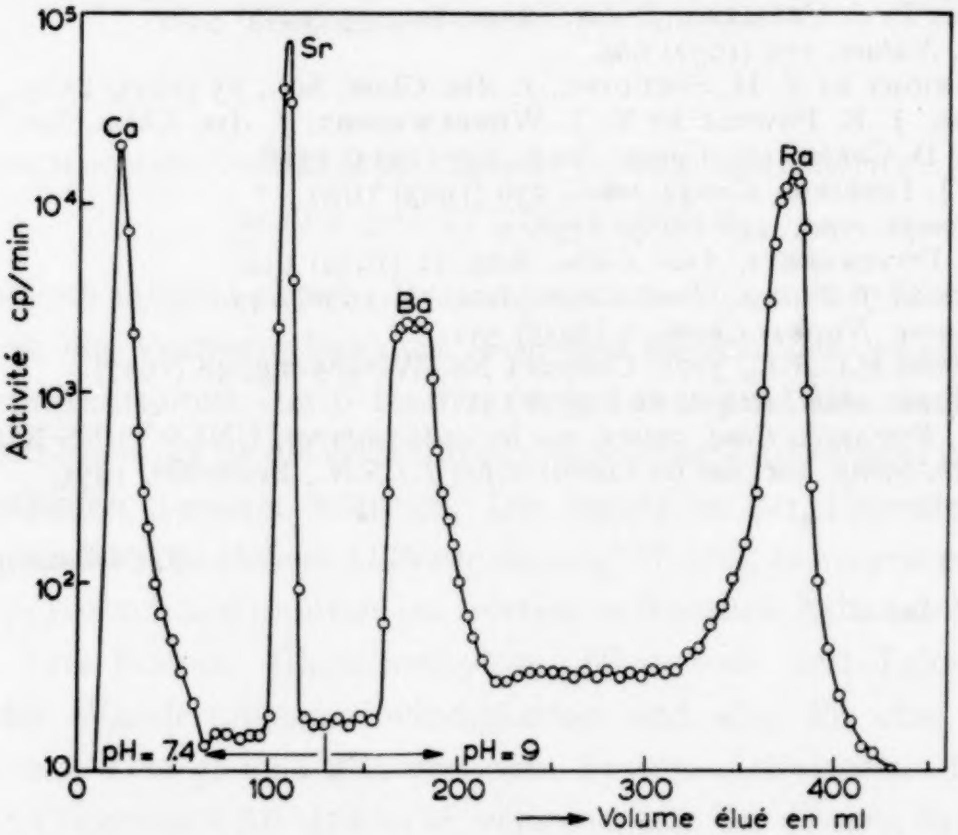

Fig. 3. Séparation chromatographique Ca–Sr–Ba–Ra à l'EDTA.

La Fig. 3 montre le chromatogramme 6 jours après l'élution au moment où les produits de filiation à vie courte ont disparu.

## REMERCIEMENT

Nous remercions l'Institut Interuniversitaire des Sciences Nucléaires dont les subsides ont permis d'effectuer ces recherches.

## RÉSUMÉ

L'EDTA est un éluant possédant de nombreuses possibilités en chimie analytique. Les auteurs ont donné quelques résultats de séparation de radioisotopes carrier-free: séparation Ba–Sr–Y–La; préparation de $^{228}$Ra à partir de thorium; séparation Ca–Sr–Ba–Ra. Tous ces résultats ont été obtenus sur Dowex 50.

## SUMMARY

EDTA is an eluant with many possibilities for analytical chemistry. The authors present the results obtained in the separation of carrier-free radioisotopes: separation

of Ba–Sr–Y–La; preparation of $^{228}$Ra from thorium; separation of Ca–Sr–Ba–Ra. All the results were obtained with Dowex 50.

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# DESOXYZUCKER

## 33. MITTEILUNG*. PAPIERCHROMATOGRAPHISCHE DIFFERENZIERUNG DER HEXAMETHYLOSEN UND IHRER 3-O-METHYL-DERIVATE**

M. T. KRAUSS, HERB. JÄGER, O. SCHINDLER UND T. REICHSTEIN
*Organisch-chemische Anstalt der Universität Basel (Schweiz)*

(Eingegangen den 20. April 1959)

### I. PAPIERCHROMATOGRAPHISCHE TRENNUNG DER HEXAMETHYLOSEN UND DEREN 3-O-METHYL-DERIVATE

Von den theoretisch möglichen acht Paaren normaler Hexamethylosen ist von jedem Paar mindestens ein Vertreter bekannt. Von den zugehörigen 3-O-Methylderivaten kennt man Repräsentanten von 6 Paaren (unbekannt sind hier noch die Allo- und die Gulo-Verbindungen). Für die Identifizierung solcher Zucker sind papierchromatographische Methoden äusserst hilfreich. Die bisher in der Literatur angegebenen Systeme sind aber für eine sichere Differenzierung*** aller Isomeren ungenügend.

HIRST UND JONES[1] bestimmten im System *n*-Butanol–Äthanol–Wasser (5 : 1 : 4) die $R_F$-Werte von Fucose, Glucomethylose, Rhamnose und Talomethylose. Die Unterschiede der Wanderungsgeschwindigkeiten sind aber für eine eindeutige Zuordnung zu gering. Das gleiche gilt von dem System Äthylacetat–Pyridin–Wasser (2 : 2 : 1), das ISHERWOOD UND JERMYN[2] verwendeten. In diesem System lässt sich höchstens Fucose sicher differenzieren. Die übrigen Hexamethylosen bilden zwei Gruppen, eine kürzer (Gulomethylose, Allomethylose und Rhamnose) und eine rascher wandernde (Altromethylose, Talomethylose und Idomethylose). Eine Zuordnung innerhalb der Gruppen ist kaum möglich. Im System *n*-Butanol–Pyridin–Wasser (3 : 2 : 1.5) waren die $R_F$-Werte von Rhamnose, Glucomethylose, Gulomethylose und Allomethylose praktisch gleich[3, 4].

Versuche zur Trennung der verschiedenen 3-O-Methylhexamethylosen beschränkten sich bisher auf Thevetose, Acofriose und Digitalose im System *n*-Butanol–Pyridin–Wasser (3 : 2 : 1.5)[4] sowie 3-O-Methyl-glucomethylose und 3-O-Methyl-altromethylose im System *n*-Butanol–Äthanol–Wasser (5 : 1 : 4)[1] und schliesslich Digitalose und Thevetose in verschiedenen Systemen durch KAISER[5]. Im zuerst erwähnten System sowie in den von KAISER empfohlenen Systemen lässt sich Digitalose abtrennen, während die Unterschiede zwischen den anderen untersuchten Zuckern zu gering sind.

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* 32. Mitteilung: O. RENKONEN UND O. SCHINDLER, *Helv. Chim. Acta*, 39 (1956) 1490.
** Auszug aus Diss. M. T. KRAUSS, Basel, die demnächst erscheint.
*** Selbstverständlich kann ein Zucker durch papierchromatographische Methoden allein nie ganz sicher identifiziert werden. Erreichbar und nötig ist aber eine eindeutige Differenzierung aller acht Raumisomeren mit normaler Kette, soweit sie bekannt sind.

Den erwähnten Systemen ist gemeinsam, dass die Zucker darin eine relativ grosse Wanderungsgeschwindigkeit besitzen. Für die Trennung nahe verwandter Stoffe sind jedoch Systeme mit kleiner Wanderungsgeschwindigkeit und dementsprechend längerer Dauer des Chromatogramms besser geeignet[6]. In Anlehnung an frühere erfolgreiche Versuche bei der Trennung der 2-Desoxy-3-O-methyl-hexamethylosen[7] haben wir nach solchen Systemen gesucht. Die drei folgenden haben sich für die Unterscheidung der sechs 3-O-Methylhexamethylosen gut bewährt*.

System I: Toluol–Methyläthylketon (1 : 2)/Wasser*.
Laufzeit 30–40 Std.

System II: Toluol–*n*-Butanol (1 : 1)/Wasser.
Laufzeit 24–28 Std.

System III: *n*-Butanol–Methyläthylketon (1 : 1)/Borsäure–Borax-Puffer*.
Laufzeit 40–50 Std.

Tabelle I enthält die relativen Wanderungsgeschwindigkeiten der 3-O-Methyl-hexamethylosen. Es geht daraus hervor, dass die Reihenfolge der Laufstrecken in den Systemen I und II gleich ist. Die Unterschiede in den Wanderungsgeschwindigkeiten genügen auch für eine sichere Unterscheidung** ausser beim Paar 3-O-Methylaltromethylose und Acovenose. Die beiden letztgenannten Zucker können aber im System III eindeutig voneinander unterschieden werden.

Bei den freien Hexamethylosen, von denen Vertreter aller acht theoretisch möglichen Isomeren-Paare bekannt sind, waren die folgenden Systeme IV und V für eine Vortrennung in Gruppen geeignet.

System IV: *n*-Butanol/Wasser.
Laufzeit 40–50 Std.

System V: Methyläthylketon/Wasser.
Laufzeit 16–23 Std.

In diesen beiden Systemen lassen sich jedoch Gulomethylose, Glucomethylose und Rhamnose einerseits sowie Idomethylose und Talomethylose andererseits, nicht sicher voneinander unterscheiden** (vgl. Tabelle II). Die Trennung dieser Zucker gelingt aber in den Systemen VI und VII, in denen wieder wie oben bei den stationären Phasen das Wasser durch Borsäure–Borax-Pufferlösung ersetzt ist.

System VI: *n*-Butanol–Äthanol (4 : 1)/Borsäure–Borax-Puffer***.
Laufzeit 28–48 Std.****

System VII: *n*-Butanol–Methyläthylketon (1 : 1)/Borsäure–Borax-Puffer.
Laufzeit 48–68 Std.

Es ist aus der Tabelle II ersichtlich, dass die geringen Unterschiede, die innerhalb der zwei Gruppen Rhamnose–Glucomethylose–Gulomethylose und Idomethylose–

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* Das Papier wurde jeweils mit der ruhenden wässrigen Phase imprägniert. Genaue Ausführung vgl. Experimenteller Teil dieser Arbeit.

** Wir betrachten zwei Zucker als eindeutig unterscheidbar, wenn sich ihre Laufstrecken im Papierchromatogramm um mindestens 10% unterscheiden.

*** Genaue Ausführung vgl. Experimenteller Teil dieser Arbeit.

**** Um die Wanderungsgeschwindigkeit zu erhöhen, wurde in diesem System Whatman No. 7 Papier an Stelle von Whatman No. 1 verwendet.

TABELLE I

LAUFSTRECKEN DER SECHS BISHER BEKANNTEN 3-O-METHYL-HEXAMETHYLOSEN BEZOGEN AUF D-THEVETOSE = 1.00

| *Zucker* | *Konfiguration der verwendeten Probe* | *Smp.* | *Laufstrecken im System* | | | *Konstellation der HO- bzw. $CH_3O$-Gruppen für die C-1 Form[8] der D-Reihe* an* | | |
|---|---|---|---|---|---|---|---|---|
| | | | *I* | *II* | *III* | *C-2* | *C-3($OCH_3$)* | *C-4* |
| Digitalose[9] | D-Galacto | 119° | 0.37 | 0.56 | 0.83 | e | e | a |
| Acofriose[10] | L-Manno | 111–114° | 0.84 | 0.74 | 1.77 | a | e | e |
| Thevetose[11] | D-Gluco | 126–129° | 1.00 | 1.00 | 1.00 | e | e | e |
| 3-O-Methyl-altromethylose[12] | D-Altro | 108–113° | 2.33 | 1.59 | 0.66 | a | a | e |
| Acovenose[13] | L-Talo | amorph | 2.49 | 1.69 | 0.83 | a | e | a |
| 3-O-Methyl-idomethylose[14] | D-Ido | amorph | 2.86–2.97 | 2.33 | 0.60 | a | a | a |

* Um die Übersicht zu erleichtern, wurde überall auf die D-Form bezogen, auch wenn nur die L-Form bekannt war. Nach der Definition von REEVES[8] ist das Spiegelbild der C-1 Form der D-Reihe als 1-C Form in der L-Reihe zu bezeichnen. Bei der D-Glucose ist also die C-1 Form und bei der L-Glucose die 1-C Form stabiler.

TABELLE II

LAUFSTRECKEN DER HEXAMETHYLOSEN BEZOGEN AUF L-RHAMNOSE = 1.00

| *Zucker* | *Smp.* | *Relative Laufstrecken* | | | | *Konstellation der HO-Gruppe für die C-1 Form der D-Reihe, bzw. 1-C Form der L-Reihe** an* | | |
|---|---|---|---|---|---|---|---|---|
| | | *IV* | *V* | *VI* | *VII** | *C-2* | *C-3* | *C-4* |
| D-Fucose | 140–146° | 0.67 | 0.68 | 0.48 | 0.46 | e | e | a |
| D-Allomethylose[15-17] | 146° | 0.91 | 1.38 | 0.78 | 0.64 | e | a | e |
| D-Glucomethylose[18] | 139–140° | 0.99 | 0.98 | 0.78 | 0.78 | e | e | e |
| L-Rhamnose | 122–126° | 1.00 | 1.00 | 1.00 | 1.00 | a | e | e |
| D-Gulomethylose[19] | 124–125° | 1.02 | 0.98 | 0.78 | 0.69 | e | a | a |
| D-Altromethylose[20] | amorph | 1.13 | 1.84 | 0.42 | 0.40 | a | a | e |
| L-Idomethylose[21] | 97–100° | 1.28 | 2.00 | 0.36 | 0.33 | a | a | a |
| L-Talomethylose[22] | 116–118° | 1.37 | 1.84 | 0.72 | 0.77 | a | e | a |

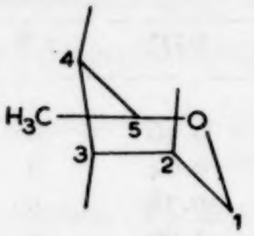
* Um die Wanderungsgeschwindigkeit zu erhöhen, wurde in diesem System Whatman No. 7 Papier an Stelle von Whatman No. 1 verwendet.

** Siehe Fussnote zu Tabelle I.

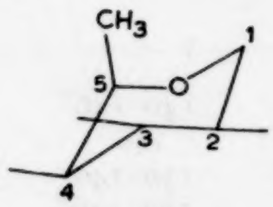
Talomethylose in den Systemen IV und V bestehen, mit Borat-Puffer als stationärer Phase in den Systemen VI und VII soweit vergrössert werden, dass eine sichere Zuordnung möglich ist. Das Paar Glucomethylose und Gulomethylose liess sich jedoch bisher nur im System VII gerade noch knapp differenzieren.

## II. VERSUCH ZUR ERKLÄRUNG DER UNTERSCHIEDE IN DEN WANDERUNGSGESCHWINDIGKEITEN

Die Reihenfolge der Wanderungsgeschwindigkeiten in den Systemen I, II, IV und V mit Wasser als stationärer Phase lässt sich ähnlich deuten, wie dies früher für die Hexosen und Pentosen geschehen ist[23]. Danach wird die Wanderungsgeschwindigkeit raumisomerer Zucker in erster Linie vom Grad ihrer Hydratisierung bestimmt[2,24,25]. Je stärker hydratisiert das Molekül ist, umso langsamer wandert es in den genannten Systemen. Da bei hexacyclischen Stoffen äquatoriale HO-Gruppen in der Regel stärker hydratisiert sind als axiale, sollte die Wanderungsgeschwindigkeit isomerer Zucker in erster Annäherung der Anzahl axialer HO-Gruppen, die sie tragen, proportional sein. In den Tabellen I und II sind die Konstellationen der verschiedenen HO-Gruppen für die C-1 Form[8] der D-Reihe* angegeben**. Es ist dabei ersichtlich, dass die angegebene Regel tatsächlich in erster Annäherung stimmt. Die Zucker mit Glucose-Konfiguration (Glucomethylose und Thevetose) verhalten sich aber auch hier wieder nicht entsprechend der Regel. Obgleich bei diesen Isomeren sämtliche HO-Gruppen und die $CH_3O$-Gruppe bei den 3-O-Methyl-derivaten äquatorial angeordnet sind, zeigen sie nicht die kürzeste Laufstrecke. Die geringste Wanderungsgeschwindigkeit kommt vielmehr auch hier wieder den Vertretern mit Galactose-Konfiguration (Fucose und Digitalose) zu***. Gewisse Differenzen gegenüber den analogen Hexosen[23] zeigen jedoch die 4 letzten Vertreter der Hexamethylosen in Tabelle II. Vor allem weist die Ido-Konfiguration in den Systemen IV und V nicht unbedingt die grösste Laufstrecke auf. Dies ist aber leicht verständlich, denn die Methylgruppe am C-Atom Nr. 5 bei den D-Hexamethylosen vermag den Pyranring nicht so stark in der C-1 Form zu fixieren wie die grössere $HOCH_2$-Gruppe der D-Hexosen****. Die D-Idomethylose dürfte in Lösung daher zu einem merklichen Prozentsatz in der 1-C Form (II) vorliegen, für die D-Formen der Altro-, Gulo- und Talomethylosen könnte dies auch noch zu einem geringeren Teil zutreffen. Vermutlich ist dies ein Grund, warum sich die Altro-, Talo- und Idomethylose im Papierchromatogramm ohne Borat-Puffer relativ schlecht trennen.



I
C-1 Form



II
1-C Form

### *Borsäure-Komplexe*

Die Komplexe, die Borsäure mit mehrwertigen Hydroxy-Verbindungen eingeht, sind eingehend untersucht[28,29] und in letzter Zeit besonders auch zur Zonen-Elektrophorese[29] benützt worden. Sie bilden sich in reversibler Reaktion. Dabei liegt in alkalifreier Borsäure-Lösung das Gleichgewicht meistens weitgehend auf der Seite der Komponenten, und erst durch Laugezusatz entstehen die Komplexe in merklicher Konzentration[30]. Je nach Reaktionsbedingungen bilden sich die 1:1-Komplexe (III) oder die spiranförmigen 2:1-Komplexe (IV), von denen nur IV viel stärker sauer sind als Borsäure selbst[28].

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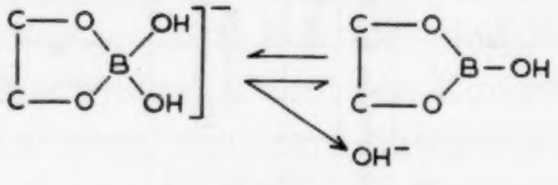
* Siehe Fussnote zu Tabelle I.

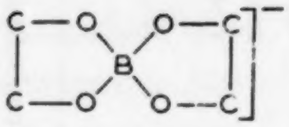
** Die HO-Gruppe am reduzierenden C-Atom wurde nicht berücksichtigt, da hier nur das Verhältnis $\alpha$: $\beta$-Form massgebend ist und dieses nicht in allen Fällen bekannt ist.

*** Ähnliche Verhältnisse herrschen auch bei den 2-Amino-2-desoxyhexosen und ihren N-Acetylderivaten. Vgl. hierzu KUHN *et al.*[26].

**** Nach PITZER UND BECKETT[27] beträgt die Energiedifferenz zwischen (a)- und (e)-Methylcyclohexan 1.8 kcal pro Mol.

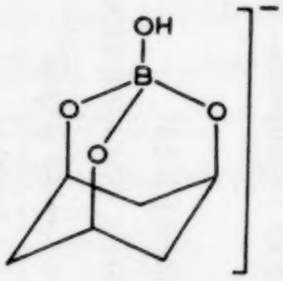


III



IV

In einzelnen Fällen wurden auch dreizähnige Komplexe (entsprechend Formel V) diskutiert, ihre Existenz scheint aber bisher nur bei Cycliten[31,32] mit *cis*-ständigen HO-Gruppen in 1,3,5 Stellung bewiesen zu sein*. Unter den von uns benutzten Bedingungen (sehr grosser Überschuss an Borat) ist vorwiegend mit 1:1-Komplexen (III) zu rechnen. Diese Bedingungen (insbesondere pH = *ca.* 8.8) entsprechen weitgehend denjenigen, die FOSTER[29, 33] für die Elektrophorese verwendete. Obgleich die Ergebnisse der Elektrophorese vermutlich nicht quantitativ auf die Verhältnisse bei der Papierchromatographie übertragen werden dürfen**, sind doch weitgehende Parallelen zu erwarten***. Nach FOSTER scheinen unter diesen Bedingungen aliphatische 1,2 und 1,3 Diole praktisch immer schwache Komplexe zu geben. Die Tendenz zur Komplexbildung steigt mit der Anzahl anwesender HO-Gruppen. Die Frage, ob bei Zuckern die freie Aldehydo-Form massgeblich an der Komplexbildung beteiligt ist, lässt er offen, da keine sicheren Beweise vorliegen. Es ist zwar bekannt, dass sich der Anteil an offenkettiger Form in alkalischer Lösung vergrössert[34]; er bleibt aber gegenüber der pyranosiden Form immer noch sehr gering. Dazu kommt, dass FOSTER bei drei raumisomeren Hexiten (Galactit, Glucit und Mannit) nur sehr wenig verschiedene Laufstrecken fand. Zur Erklärung der Laufstrecken unserer Zucker im



V

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* FOSTER (Ref.[29], S. 101) macht darauf aufmerksam, dass die Bildung eines dreizähnigen Komplexes an den HO-Gruppen von C3, C5 und C6 in gewissen D-Gluco-furanosiden möglich wäre.

** Bei der Elektrophorese dürfte schon ein kleiner Anteil des Spirans IV wegen seiner viel stärkeren Ladung die Laufgeschwindigkeit erheblich vergrössern. Falls sich das Gleichgewicht zwischen III und IV jeweils rasch genug einstellt, sollte keine Aufspaltung in zwei Zonen erfolgen. Bei der Papierchromatographie würde sich ein kleiner Anteil an IV viel weniger auswirken.

*** In reiner Borsäure, wie sie BÖESEKEN[28] meistens für seine Messungen verwendet hat, herrschen stark abweichende Gesetzmässigkeiten.

## TABELLE III

### VERKLEINERUNG DES $R_F$-WERTES MIT BORAT-PUFFER ALS STATIONÄRER PHASE UND MÖGLICHKEITEN DER KOMPLEXBILDUNG

| *Zucker (Konfiguration)* | *Verzögerungs-faktor* | *Mögliche Komplexbildung in der pyranosiden C-1 Form der* D-*Reihe** | | | | *Mögliche Komplexbildung in der pyranosiden 1-C Form der* D-*Reihe** | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *e–a Komplex* | | *a–a Komplex* | | *e–a Komplex* | | *a–a Komplex* | |
| | | *Beteiligte HO-Gruppen* | *Stellung zu* $-OCH_3$ | *Beteiligte HO-Gruppen* | *Stellung zu* $-OCH_3$ | *Beteiligte HO-Gruppen* | *Stellung zu* $-OCH_3$ | *Beteiligte HO-Gruppen* | *Stellung zu* $OCH_3$ |
| Digitalose (Galacto) | 3.2 | 1α–2 | *trans* | — | — | 1α–2 | *trans* | — | — |
| Acofriose (Manno) | 1.42 | 1β–2 | *cis* | — | — | 1β–2 | *cis* | — | — |
| Thevetose (Gluco) | 3.80 | 1α–2 | *trans* | — | — | 1α–2 | *trans* | 2–4 | *trans* |
| 3-O-Methyl-altromethylose (Altro) | 6.15 | 1β–2 | *trans* | — | — | 1β–2 | *trans* | — | — |
| Acovenose (Talo) | 4.64 | 1β–2 | *cis* | 2–4 | *cis* | 1β–2 | *cis* | — | — |
| 3-O-Methyl-idomethylose (Ido) | 6.84 | 1β–2 | *trans* | 2–4 | *trans* | 1β–2 | *trans* | — | — |

* Um die Übersicht zu erleichtern, wurde überall auf die D-Form bezogen, auch wenn nur die L-Form bekannt war. Nach der Definition von REEVES[8] ist das Spiegelbild der C-1 Form der D-Reihe als 1-C Form in der L-Reihe zu bezeichnen. Bei der D-Glucose ist also die C-1 Form und bei der L-Glucose die 1-C Form stabiler.

## TABELLE IV

### VERZÖGERUNGSFAKTOREN UND MÖGLICHKEITEN ZUR KOMPLEXBILDUNG BEI HEXAMETHYLOSEN

| *Zucker* | *Relativer Verzögerungs-faktor bezogen auf Rhamnose** | *Mögliche Komplexbildung in der pyranosiden C-1 Form der* D-*Reihe*** | | | | *Mögliche Komplexbildung in der pyranosiden 1-C Form der* D-*Reihe*** | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *e–a Komplexe* | | *a–a Komplexe* | | *e–a Komplexe* | | *a–a Komplexe* | |
| | | *Beteiligte HO-Gruppen* | *Relative Lage ev. gleichz. mögl. Kompl. zueinander* | *Beteiligte HO-Gruppen* | *Relative Lage ev. gleichz. mögl. Kompl. zueinander* | *Beteiligte HO-Gruppen* | *Relative Lage der Komplexe zueinander* | *Beteiligte HO-Gruppen* | *Relative Lage der Komplexe zueinander* |
| Rhamnose | 1.00* | 1β–2, 2–3 | — | — | — | 1β–2, 2–3 | — | 1β–3 | — |
| Allomethylose | 1.17 | 1α–2, 2–3, 3–4 | *cis* | 1α–3 | — | 1α–2, 2–3, 3–4 | *cis* | 2–4 | — |
| Gulomethylose | 1.31 | 1α–2, 2–3 | — | 1α–3 | — | 1α–2, 2–3 | — | — | — |
| Fucose | 1.40 | 1α–2, 3–4 | *trans* | — | — | 1α–2, 3–4 | *trans* | 1β–3 | — |
| Glucomethylose | 1.43 | 1α–2 | — | — | — | 1α–2 | — | 1β–3, 2–4 | *trans* |
| Talomethylose | 1.78 | 1β–2, 2–3, 3–4 | *cis* | 2–4 | — | 1β–2, 2–3, 3–4 | *cis* | 1β–3 | — |
| Altromethylose | 2.82 | 1β–2, 3–4 | *trans* | 1α–3 | — | 1β–2, 3–4 | *trans* | — | — |
| Idomethylose | 3.88 | 1β–2 | — | 1α–3, 2–4 | *trans* | 1β–2 | — | — | — |

* Die Methode der Berechnung der Faktoren bedingt, dass Rhamnose den Wert 1.00 erhält. Dies bedeutet aber nicht, dass Rhamnose mit Borat-Ionen keinen Komplex bildet, sondern nur, dass der Komplex am schwächsten ist.

** Siehe Fussnote zu Tabelle III.

Papierchromatogramm wollen wir uns daher auf die pyranosiden Ringformen beschränken*, da sie in Lösung bestimmt dominieren.

Zunächst kann aus den Papierchromatogrammen, wie erwartet, entnommen werden, dass die Komplexbildung die Wanderungsgeschwindigkeit des Zuckers verkleinert. Die Verzögerung ist umso grösser, je günstiger die Bedingungen für die Komplexbildung liegen. Nach FOSTER[29] sind bei Pyranosen zwei benachbarte *cis*-ständige HO-Gruppen nötig, damit ein Komplex entstehen kann**. Der Abstand zwischen den O-Atomen von zwei äquatorialen *trans*-ständigen HO-Gruppen in Cyclohexan beträgt 2.83 Å[36,37]; dieser Abstand ist offenbar noch zu gross, um die Komplexbildung mit Borat zu ermöglichen. Bei benachbarten *cis*-ständigen Gruppen ist dieser Abstand zwar praktisch gleich gross, lässt sich aber durch eine leichte Ausflachung der Sesselform des 6-Ringes verkleinern***. Aus den zwei Tabellen III und IV glauben wir auch schliessen zu müssen, dass zwei $\beta$-ständige *cis*-axiale HO-Gruppen zur Komplexbildung befähigt sind. Der Abstand ihrer O-Atome beträgt für die Sesselform des Cyclohexans 2.51 Å[36,37]. Die dreizähnigen Komplexe gewisser Cyclite (III) müssen ja auch entsprechende Bindungen enthalten.

Um einen brauchbaren Vergleich zwischen Laufstrecke und Komplexbildung zu erhalten, haben wir die Laufstrecken der 3-O-Methyl-hexamethylosen (Tabelle I) im System III mit den Werten verglichen, die beim Ersatz des Borats durch einen Na-phosphat-Puffer von gleichem pH und möglichst gleicher Molarität erhalten wurden (vgl. Tabelle III). Wir bezeichnen hier den Quotienten

$$\frac{\text{Laufstrecke im Phosphatsystem}}{\text{Laufstrecke im Boratsystem}} = \text{Verzögerungsfaktor.}$$

Diesen Verzögerungsfaktor betrachten wir als brauchbares Mass für die Komplexbildung.

Bei den Hexamethylosen haben wir auf die Bestimmung der Laufstrecken im Phosphatsystem verzichtet und direkt die Laufstrecken der Systeme IV und VI miteinander verglichen. Die so erhaltenen Verzögerungsfaktoren (vgl. Tabelle IV) sind durch den "Salzeffekt" verfälscht, aus diesem Grunde wurden in Tabelle IV keine Absolutwerte sondern nur Relativwerte angegeben****.

### *Besprechung der 3-O-Methyl-hexamethylosen*

Bei den Zuckern der Tabelle III sind e-a-Komplexe (äquatorial-axial, also solche zwischen *cis*-ständigen benachbarten HO-Gruppen) nur unter Beteiligung der Hydroxyle an den ersten zwei C-Atomen möglich. Für die C-1 Form der D-Reihe ergibt sich

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[*] FOSTER fand bei Aldopentosen und Aldohexosen keine sicheren Anhaltspunkte dafür, dass die furanoside Form massgeblich an der Komplexbildung beteiligt wäre.

[**] Bei Hexosen kann sich auch die $HOCH_2$-Gruppe (C6) an der Komplexbildung beteiligen, die in unseren Zuckern fehlt. Hier herrschen daher andere Verhältnisse. Über sterische Beeinflussung dieser 6-Ring-Komplexbildung vgl. BARKER *et al.*[35].

[***] Es ist weniger Energie nötig um die Sesselform eines 6-Ringes auszuflachen als um sie noch mehr zu knicken.

[****] Die relativen Laufstrecken der Zucker ändern sich nicht merklich, wenn man von Wasser zu Phosphat übergeht.

für die Acovenose und für die 3-O-Methyl-idomethylose ausserdem noch die Möglichkeit zur Bildung eines a-a-Komplexes (di-axial zwischen den HO-Gruppen an den C-Atomen 2 und 4). Diese zwei Zucker zeigen auch einen besonders grossen Verzögerungsfaktor*. Es ist ferner zu beachten, dass die Ausbildung eines Komplexes durch eine benachbarte *cis*-ständige $CH_3O$-Gruppe erschwert wird[33]. Eine zum Boratkomplex *trans*-ständige $CH_3O$-Gruppe ist ohne Einfluss. Der Vergleich der Zahlen zeigt diesen Effekt deutlich. So besitzt z.B. Acofriose ein gleich günstig gelegenes Paar von HO-Gruppen wie 3-O-Methyl-altromethylose, bei der Acofriose ist aber die Komplexbildung durch die *cis*-ständige $CH_3O$-Gruppe gehemmt. Ähnlich ist der Unterschied zwischen Acovenose und 3-O-Methyl-idomethylose erklärbar, die beide je zwei Paare von günstig gelagerten HO-Gruppen besitzen. Bei der Acovenose stört die $CH_3O$-Gruppe die Ausbildung beider Komplexe. Der im Vergleich zu Thevetose und Digitalose sehr grosse Verzögerungsfaktor von 3-O-Methyl-altromethylose lässt sich aber mit diesem Einfluss der $CH_3O$-Gruppe allein nicht erklären. Alle drei Zucker können nämlich nur einen e-a-Komplex (an den C-Atomen 1 und 2) bilden, der in allen drei Fällen *trans*-ständig zur $CH_3O$-Gruppe angeordnet ist. Hingegen haben wir bisher den Einfluss des in Lösung vorhandenen Gleichgewichtes zwischen den α- und β-Formen gar nicht berücksichtigt. Bei der Thevetose und bei der Digitalose ist jeweils nur das α-Isomere, dessen HO-Gruppe am ersten C-Atom in der C-1 Form der D-Reihe axial gelegen ist, zur Komplexbildung befähigt. Auf Grund der Drehung enthalten Thevetose *ca.* 35% und Digitalose *ca.* 30% der zur Komplexbildung günstigen α-Form. Bei der 3-O-Methyl-altromethylose ist für die Komplexbildung nur die β-Form geeignet. Benützt man hier wieder den Vergleich der Drehung des Gleichgewichtsgemisches und der reinen Methyl-glykoside** als Mass für den Gehalt an β-Form, so kommt man auf *ca.* 53%. Wir zweifeln aber, ob diese Art der Rechnung hier zulässig ist. D-Thevetose und D-Digitalose sollten in Analogie zu D-Glucose und D-Galactose nach REEVES[8] in Lösung praktisch ausschliesslich in der C-1 Form vorliegen. Bei 3-O-Methyl-D-altromethylose dürfte in Lösung jedoch ein Gleichgewicht zwischen C-1 und 1-C Form vorhanden sein, die stark verschiedene Drehung besitzen müssen, so dass man aus der Drehung nicht mehr so gut auf den Gehalt an α- und β-Form zurückschliessen kann. Es ist aber aus dem räumlichen Bau leicht ersichtlich, dass für die C-1 Form das β-Isomere stark begünstigt sein muss. Das axiale α-Isomere ist nämlich durch die ebenfalls axiale Methoxylgruppe in 3-Stellung besonders stark benachteiligt. Hier dürfte die Methoxylgruppe somit die bevorzugte Ausbildung der für die Komplexbildung günstigsten β-Form erzwingen.

Dieselben Verhältnisse finden wir auch bei der 3-O-Methyl-idomethylose, nur dass hier ausserdem noch die Möglichkeit zur Ausbildung eines a–a-Komplexes gegeben ist. Bei der Thevetose und bei der Digitalose liegt die $CH_3O$-Gruppe äquatorial und sollte demnach keinen merklichen Einfluss auf das α, β-Gleichgewicht haben. Bei den zwei noch unbekannten Isomeren, der 3-O-Methyl-allomethylose sowie der

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* Bei der Altromethylose ist er noch grösser, vielleicht weil die Komplexbildung bei der Acovenose *in beiden Fällen* durch die $CH_3O$-Gruppe gehemmt ist.

** Zur Abschätzung dienten die Werte der entsprechenden Hexosen.

3-O-Methyl-gulomethylose, die beide in der C-1 Form der D-Reihe axiale $CH_3O$-Gruppen tragen, sollte die Ausbildung der für die Komplexbildung günstigeren α-Isomeren stark erschwert sein. Dabei wäre auch wieder zu berücksichtigen, dass 3-O-Methyl-gulomethylose in Lösung teilweise in 1-C Form vorliegen dürfte.

Die Papierchromatogramme mit Boratpuffer deuten darauf hin, dass die D-Formen aller 6 hier untersuchten Zucker im Boratsystem vorwiegend in der C-1 Form vorliegen, wobei sogar die sonst instabilen C-1 Formen der 3-O-Methyl-altromethylose und besonders der 3-O-Methyl-idomethylose (Formel I) durch die Komplexbildung weitgehend stabilisiert werden. Bei der Idomethylose sollte in der 1-C Form der D-Reihe (Formel II) wegen der axialen Methylgruppe an C5 die für die Komplexbildung benötigte β-Form sehr stark behindert sein, so dass für die 1-C Form der D-Reihe nur ein sehr geringer Verzögerungsfaktor zu erwarten gewesen wäre*.

### *Besprechung der Hexamethylosen*

In Tabelle IV sind die Verzögerungsfaktoren sowie sämtliche Möglichkeiten der Komplexbildung für e-a- und a-a-Komplexe für die C-1 und die 1-C Formen der D-Reihe zusammengestellt.

Wenn die Komplexbildung an einem Paar von HO-Gruppen die weitere Komplexbildung an einem zweiten Paar nicht hindern oder verunmöglichen würde, sollte derjenige Zucker den grössten Verzögerungsfaktor aufweisen, der die meisten Paare geeigneter HO-Gruppen enthält. Dies ist offensichtlich nicht der Fall, und der Grund ist auch leicht ersichtlich. Wenn z.B. bei der C-1 Form der D-Allomethylose, welche total 4 geeignete Paare von HO-Gruppen enthält, an 2–3 Komplexbildung erfolgt, so kann an 1α–2, 3–4 und 1β–3 keine weitere Komplexbildung stattfinden. Bei Fucose, die nur zwei geeignete Paare enthält, können prinzipiell beide gleichzeitig eine Komplexbildung eingehen. Komplexe mit zwei Boratmolekülen kann auch Altromethylose bilden, die insgesamt nur 3 geeignete Paare von HO-Gruppen enthält. Dies macht es verständlich, dass Fucose und Altromethylose einen grösseren Verzögerungsfaktor zeigen als Allomethylose.

Aus den Werten der Tabelle IV lässt sich weiter ableiten, dass Komplexe mit zwei Boratgruppen pro Zucker nur dann möglich sind, wenn sie sich gegenseitig nicht behindern. Dies setzt voraus, dass sie *trans*-ständig sind. Der merklich grössere Verzögerungsfaktor von Fucose, die nur zwei Paare geeigneter HO-Gruppen enthält im Vergleich zu Rhamnose (2 Paare), Allomethylose (4 Paare!) und Gulomethylose (3 Paare) wird so leicht erklärbar. Wir glauben daher auch, dass der besonders grosse Verzögerungsfaktor bei der Idomethylose durch die Bildung des Komplexes mit zwei *trans*-ständigen Boratgruppen an 1α–3 und 2–4 der C-1 Form der D-Reihe (Formel I) bedingt ist und dass diese sonst unstabile Form durch diese Komplexbildung stabilisiert wird. Für die 1-C Form der D-Reihe wäre, wie aus Tabelle IV ersichtlich, nur ein 1β–2-Komplex und damit ein viel kleinerer Verzögerungsfaktor zu erwarten.

Die beobachteten Verzögerungsfaktoren lassen vermuten, dass auch von den für die Acovenose und für die 3-O-Methyl-idomethylose ausserdem noch die Möglichkeit zur Bildung eines a-a-Komplexes (di-axial zwischen den HO-Gruppen an den C-Atomen 2 und 4). Diese zwei Zucker zeigen auch einen besonders grossen Verzögerungsfaktor*. Es ist ferner zu beachten, dass die Ausbildung eines Komplexes durch eine benachbarte *cis*-ständige $CH_3O$-Gruppe erschwert wird[33]. Eine zum Boratkomplex *trans*-ständige $CH_3O$-Gruppe ist ohne Einfluss. Der Vergleich der Zahlen zeigt diesen Effekt deutlich. So besitzt z.B. Acofriose ein gleich günstig gelegenes Paar von HO-Gruppen wie 3-O-Methyl-altromethylose, bei der Acofriose ist aber die Komplexbildung durch die *cis*-ständige $CH_3O$-Gruppe gehemmt. Ähnlich ist der Unterschied zwischen Acovenose und 3-O-Methyl-idomethylose erklärbar, die beide je zwei Paare von günstig gelagerten HO-Gruppen besitzen. Bei der Acovenose stört die $CH_3O$-Gruppe die Ausbildung beider Komplexe. Der im Vergleich zu Thevetose und Digitalose sehr grosse Verzögerungsfaktor von 3-O-Methyl-altromethylose lässt sich aber mit diesem Einfluss der $CH_3O$-Gruppe allein nicht erklären. Alle drei Zucker können nämlich nur einen e-a-Komplex (an den C-Atomen 1 und 2) bilden, der in allen drei Fällen *trans*-ständig zur $CH_3O$-Gruppe angeordnet ist. Hingegen haben wir bisher den Einfluss des in Lösung vorhandenen Gleichgewichtes zwischen den α- und β-Formen gar nicht berücksichtigt. Bei der Thevetose und bei der Digitalose ist jeweils nur das α-Isomere, dessen HO-Gruppe am ersten C-Atom in der C-1 Form der D-Reihe axial gelegen ist, zur Komplexbildung befähigt. Auf Grund der Drehung enthalten Thevetose *ca.* 35% und Digitalose *ca.* 30% der zur Komplexbildung günstigen α-Form. Bei der 3-O-Methyl-altromethylose ist für die Komplexbildung nur die β-Form geeignet. Benützt man hier wieder den Vergleich der Drehung des Gleichgewichtsgemisches und der reinen Methyl-glykoside** als Mass für den Gehalt an β-Form, so kommt man auf *ca.* 53%. Wir zweifeln aber, ob diese Art der Rechnung hier zulässig ist. D-Thevetose und D-Digitalose sollten in Analogie zu D-Glucose und D-Galactose nach REEVES[8] in Lösung praktisch ausschliesslich in der C-1 Form vorliegen. Bei 3-O-Methyl-D-altromethylose dürfte in Lösung jedoch ein Gleichgewicht zwischen C-1 und 1-C Form vorhanden sein, die stark verschiedene Drehung besitzen müssen, so dass man aus der Drehung nicht mehr so gut auf den Gehalt an α- und β-Form zurückschliessen kann. Es ist aber aus dem räumlichen Bau leicht ersichtlich, dass für die C-1 Form das β-Isomere stark begünstigt sein muss. Das axiale α-Isomere ist nämlich durch die ebenfalls axiale Methoxylgruppe in 3-Stellung besonders stark benachteiligt. Hier dürfte die Methoxylgruppe somit die bevorzugte Ausbildung der für die Komplexbildung günstigsten β-Form erzwingen.

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* Die verschiedenen, von REEVES UND BLOUIN[38] abgeleiteten Wannen-Formen wurden vernachlässigt.

Dieselben Verhältnisse finden wir auch bei der 3-O-Methyl-idomethylose, nur dass hier ausserdem noch die Möglichkeit zur Ausbildung eines a–a-Komplexes gegeben ist. Bei der Thevetose und bei der Digitalose liegt die $CH_3O$-Gruppe äquatorial und sollte demnach keinen merklichen Einfluss auf das α, β-Gleichgewicht haben. Bei den zwei noch unbekannten Isomeren, der 3-O-Methyl-allomethylose sowie der

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Die Papierchromatogramme mit Boratpuffer deuten darauf hin, dass die D-Formen aller 6 hier untersuchten Zucker im Boratsystem vorwiegend in der C-1 Form vorliegen, wobei sogar die sonst instabilen C-1 Formen der 3-O-Methyl-altromethylose und besonders der 3-O-Methyl-idomethylose (Formel I) durch die Komplexbildung weitgehend stabilisiert werden. Bei der Idomethylose sollte in der 1-C Form der D-Reihe (Formel II) wegen der axialen Methylgruppe an C5 die für die Komplexbildung benötigte β-Form sehr stark behindert sein, so dass für die 1-C Form der D-Reihe nur ein sehr geringer Verzögerungsfaktor zu erwarten gewesen wäre*.

*Besprechung der Hexamethylosen*

In Tabelle IV sind die Verzögerungsfaktoren sowie sämtliche Möglichkeiten der Komplexbildung für e-a- und a-a-Komplexe für die C-1 und die 1-C Formen der D-Reihe zusammengestellt.

Wenn die Komplexbildung an einem Paar von HO-Gruppen die weitere Komplexbildung an einem zweiten Paar nicht hindern oder verunmöglichen würde, sollte derjenige Zucker den grössten Verzögerungsfaktor aufweisen, der die meisten Paare geeigneter HO-Gruppen enthält. Dies ist offensichtlich nicht der Fall, und der Grund ist auch leicht ersichtlich. Wenn z.B. bei der C-1 Form der D-Allomethylose, welche total 4 geeignete Paare von HO-Gruppen enthält, an 2–3 Komplexbildung erfolgt, so kann an 1α–2, 3–4 und 1β–3 keine weitere Komplexbildung stattfinden. Bei Fucose, die nur zwei geeignete Paare enthält, können prinzipiell beide gleichzeitig eine Komplexbildung eingehen. Komplexe mit zwei Boratmolekülen kann auch Altromethylose bilden, die insgesamt nur 3 geeignete Paare von HO-Gruppen enthält. Dies macht es verständlich, dass Fucose und Altromethylose einen grösseren Verzögerungsfaktor zeigen als Allomethylose.

Aus den Werten der Tabelle IV lässt sich weiter ableiten, dass Komplexe mit zwei Boratgruppen pro Zucker nur dann möglich sind, wenn sie sich gegenseitig nicht behindern. Dies setzt voraus, dass sie *trans*-ständig sind. Der merklich grössere Verzögerungsfaktor von Fucose, die nur zwei Paare geeigneter HO-Gruppen enthält im Vergleich zu Rhamnose (2 Paare), Allomethylose (4 Paare!) und Gulomethylose (3 Paare) wird so leicht erklärbar. Wir glauben daher auch, dass der besonders grosse Verzögerungsfaktor bei der Idomethylose durch die Bildung des Komplexes mit zwei *trans*-ständigen Boratgruppen an 1α–3 und 2–4 der C-1 Form der D-Reihe (Formel I) bedingt ist und dass diese sonst unstabile Form durch diese Komplexbildung stabilisiert wird. Für die 1-C Form der D-Reihe wäre, wie aus Tabelle IV ersichtlich, nur ein 1β–2-Komplex und damit ein viel kleinerer Verzögerungsfaktor zu erwarten.

Die beobachteten Verzögerungsfaktoren lassen vermuten, dass auch von den anderen drei Zuckern, deren C-1 Form der D-Reihe in Wasser teilweise mit der 1-C Form in Gleichgewicht steht (Altromethylose, Gulomethylose und Talomethylose in ungefähr abnehmender Reihenfolge), die Altromethylose und Talomethylose in Boratlösung vorwiegend auch in der C-1 Form (als Komplex stabilisiert) vorliegen. Für die Gulomethylose ist dies dagegen unsicher.

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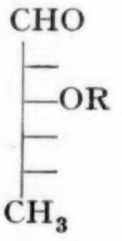
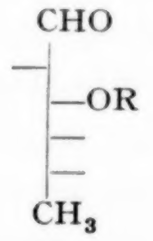
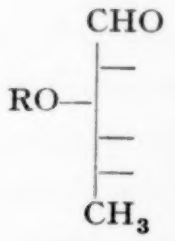
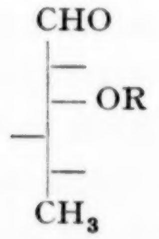
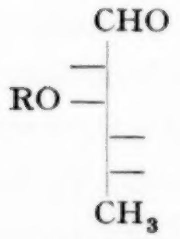
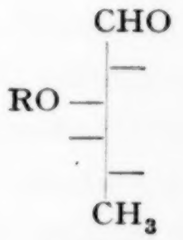
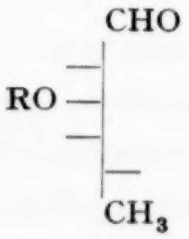
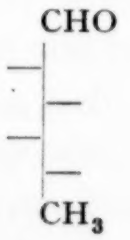
* Die verschiedenen, von REEVES UND BLOUIN[38] abgeleiteten Wannen-Formen wurden vernachlässigt.

| Konfig. | Allo | Altro | Gluco |
|---|---|---|---|
| Freie Hexamethylose<br>(R = H) | I. Allomethylose<br>F. 146°<br>$[\alpha]_D = -1.0^\circ$[16] | II. Altromethylose<br>amorph<br>$[\alpha]_D = +16^\circ$[20] | III. Glucomethylose<br>F. 139–140°<br>$[\alpha]_D = +29.7^\circ$[18] |
| 3-O-Methylderivat<br>($R = CH_3$) | IX. Unbekannt | X. 3-O-Methyl-altromethylose<br>F. 108–113°<br>$[\alpha]_D = +22.3^\circ$[12] | XI. Thevetose<br>F. 110–120°<br>$[\alpha]_D = +35.5^\circ$[39] |

| Konfig. | Gulo | Manno | Galacto |
|---|---|---|---|
| Freie Hexamethylose<br>(R = H) | IV. Gulomethylose<br>F. 124–125°<br>$[\alpha]_D = -35.7^\circ$[19] | V. Rhamnose*<br>F. 122–126°<br>$[\alpha]_D = -8.9^\circ$ | VI. Fucose<br>F. 140–146°<br>$[\alpha]_D = +76.3^\circ$ |
| 3-O-Methylderivat<br>($R = CH_3$) | XII. Unbekannt | XIII. Acofricose*<br>F. 111–114°<br>$[\alpha]_D = -39.1^\circ$[10] | XIV. Digitalose<br>F. 119°<br>$[\alpha]_D = +106^\circ$[9] |

| Konfig. | Talo | Ido |
|---|---|---|
| Freie Hexamethylose<br>(R = H) | VII. Talomethylose*<br>F. 116–118°<br>$[\alpha]_D = +18.9^\circ$[22] | VIII. Idomethylose*<br>F. 95–98°<br>$[\alpha]_D = +26^\circ$[21] |
| 3-O-Methylderivat<br>($R = CH_3$) | XV. Acovenose*<br>amorph[13] | XVI. 3-O-Methyl-idomethylose<br>amorph<br>$[\alpha]_D = +22.1^\circ$[14] |

Formeln der hier untersuchten Zucker mit Angaben der Smp. Die angegebenen optischen Drehungen geben die spez. Enddrehung in Wasser. Von den mit * bezeichneten Zuckern ist nur die L-Form bekannt. Wir geben für die D-Form denselben Smp. und die umgekehrte Drehung.

## EXPERIMENTELLER TEIL

Die Chromatogramme wurden zwischen 23° und 25° Raumtemperatur absteigend ausgeführt.

Für das System VII (*n*-Butanol–Methyläthylketon (1:1)/Borat-Pufferlösung) wurde Whatman No. 7 Papier verwendet. Alle übrigen Chromatogramme wurden auf Papier No. 1 durchgeführt.

Zur Sättigung der mobilen Phase wurde diese während 3 Std. mit gleichem Volumen stationärer Phase geschüttelt und nach der Trennung der Schichten die mobile Phase abgetrennt.

Wenn Wasser als stationäre Phase diente, wurden die Papiere durch eine Mischung von Wasser–Aceton (1:2) gezogen und dann zwischen Filtrierpapier ausgepresst. Hierauf wurden die Substanzproben aufgetragen, und dann das Papier so lange an der Luft getrocknet, bis die Gewichtszunahme gegenüber dem trockenen, unbehandelten Papier 50% betrug. Im Falle von Pufferlösung als stationärer Phase wurde das Papier durch die Lösung gezogen, zwischen Filterpapier abgepresst und nach Auftragen der Substanzprobe so lange an der Luft trocknen gelassen, bis auch hier die Gewichtszunahme 50% betrug. Nach dem Einhängen der Papiere in den Trog wurde unmittelbar mit dem Entwickeln begonnen.

Nach Abschluss der Chromatographie wurden die Papiere so lange bei Raumtemperatur einem schwachen Luftstrom ausgesetzt, bis sie sich trocken anfühlten (*ca.* 20 Min.). Zum Lokalisieren der Zucker diente entweder Blautetrazolium[40] (durch die Reagenslösung gezogen) oder Anilinphtalat nach PARTRIDGE[41] (besprüht). Um das Zerreissen der mit dem Reagens imprägnierten Papiere zu vermeiden, wurde das Papier auf dünne, entsprechend dimensionierte Glasplatten gelegt und durch schwache Erwärmen derselben die Farbreaktionen erzeugt.

Herstellung der Pufferlösung: 3.814 g Borax ($Na_2B_4O_7 \cdot 10H_2O$) und 0.618 g Borsäure wurden in je 100 ml Wasser gelöst; für die Bereitung der stationären Phase wurden gleiche Volumteile der beiden Lösungen vermischt; pH (potentiometrisch bestimmt): 8.94.

Herstellung der Phosphatlösung: 1.166 g 84% $H_3PO_4$ (= 0.685 ml) wurden mit 20.8 ml 0.3 *N* NaOH versetzt; pH (potentiometrisch bestimmt): 8.8.

## DANK

Der eine von uns (M.T.K.) dankt dem Research Grant Committee Eli Lilly and Company, Indianapolis, für einen Beitrag an die Kosten der Arbeit.

## ZUSAMMENFASSUNG

Es werden Methoden zur papierchromatographischen Unterscheidung der Hexamethylosen sowie deren 3-O-Methyl-derivate beschrieben. Die $R_F$-Werte, die erhalten werden, wenn eine Natriumboratlösung als stationäre Phase diente, wurden mit den stereochemischen Faktoren der Komplexbildung in Beziehung gebracht.

## SUMMARY

Methods are described for the paper-chromatographic separation of hexamethyloses and of their 3-O-methyl derivatives. The $R_F$ values obtained when a sodium borate solution was used as stationary phase were correlated with the stereochemical factors involved in complex formation.

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# THE FRACTIONATION AND ESTIMATION OF FREE AMINO ACIDS IN SERUM

E. R. COOK AND MOLLIE LUSCOMBE

*Rheumatism Research Unit, Royal National Hospital for Rheumatic Diseases, Bath (Great Britain)*

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Recent reports[1] have shown that there is an altered amino acid metabolism in rheumatoid arthritis, and it was considered of importance to study this abnormality in greater detail. However, no suitable chemical method for the quantitative estimation of amino acids in blood was available, other than the elegant but complex technique of MOORE AND STEIN[2], and consequently a simpler method has been developed in this department.

Acting on a suggestion by D. BIDMEAD, the analytical problem was divided into three parts, (a) removal of protein and salts from the serum, (b) separation into individual amino acids and (c) quantitative estimation of each amino acid.

BOULANGER, BISERTE AND COURTOT[3] have shown that the usual reagents used for precipitating proteins can cause losses of numerous amino acids, and also excess reagent must be removed[4]. Preliminary experiments with some other deproteinising agents, including picric acid followed by Dowex-2[5], alcohol[6], acetic acid at controlled pH[7], and the extraction of dried plasma with acid acetone[8] or butanol–phenol mixtures, gave extracts which were not suitable for our purposes. These methods were also criticised by AWAPARA AND SATO[9]. JIRGL[10] has employed a mixture of alcohol and acetone followed by ethanol to prepare blood extracts for chromatography, but only 12 amino acids were shown to be present in such extracts.

STEIN AND MOORE[11], and FLOCK *et al.*[12] have shown that the process of electrolytic desalting, used by many authors, may cause losses of certain amino acids. An alternative chemical technique has been employed by MCCOLLUM AND RIDER[13] who used acetone containing *dl*-camphorsulphonic acid to extract the amino acids from salt, followed by saturation with ammonia gas to liberate the dissolved acids.

However, ion exchange resins can be used for the simultaneous removal of both salts and protein (see *e.g.* REDFIELD[14], CARSTEN[15], PIEZ *et al.*[16]). Recently COCHRANE *et al.*[17] have passed serum through a column of Zeocarb 225(H) which adsorbed the amino acids but allowed the proteins, sugars, acids and other compounds to be washed through. Elution with 5 *M* ammonia extracted the amino acids free of salts. Unfortunately no detailed practical account was given. We have investigated this system and have found that the extracts obtained are suitable for subsequent fractionation by electrophoresis.

Two-way paper chromatography offers a convenient way of separating complex mixtures of amino acids, but the spots are often diffuse and may consist of overlapping compounds (see *e.g.* GORDON AND NARDI[8], FLOCK *et al.*[12]). It was considered a better approach technically to separate the mixed amino acids into groups by electrophoresis followed by the analysis of each group by descending paper chromatography.

Initial experiments showed that high voltage electrophoresis in barbitone buffer (pH 8.2) would separate serum extracts into 4 groups of amino acids, but the technique was discarded partly through practical difficulties and partly due to the necessity for removing the barbitone buffer prior to paper chromatography. Eventually a method was adopted which employed electrophoresis in $N$ acetic acid buffer (after DURRUM[18]). By this means it was found possible to split the free amino acids of serum into 6 groups which could then be located on the paper by a non-destructive fluorescence method (COOK AND LUSCOMBE[19]). Each group was then separated into its component amino acids by descending paper chromatography employing a solution of butanol, ethanol, ethyl acetate and dilute acetic acid. The individual acids were measured as the stable pink complexes formed on addition of cadmium sulphate to the blue colour produced by ninhydrin and amino acids[20]. Salts of zinc, nickel, copper and cobalt were substituted for cadmium sulphate but were not satisfactory.

This paper presents a detailed account of the method together with some preliminary results from its application to normal sera.

## MATERIALS AND METHOD

### *Materials*

*Zeocarb 225H* was prepared by washing approximately 400 g (60–80 mesh) with 1 l of 6 $N$ ammonia followed by 5 l of distilled water. The material was then treated with 100 ml of 40 % HCl, 300 ml of 20 % HCl, and washed with water until the washings were free from chloride. The resin was stored under water until required, but some decomposition occurs after 3–4 weeks, as shown by a yellow colour in the supernatant water.

*Power-pack* for high voltage electrophoresis (1350 V) was designed and built by A. J. S. McMillan, 5 Oakfield Road, Bristol 8, Great Britain.

### *Method*

*Extraction of amino acids from serum.* Practical experience showed that consistent results were not obtained by shaking the serum with added resin, and consequently the following column technique was adopted. A column of Zeocarb 225(H), 5 cm long, 0.4 cm diameter, is prepared by pouring an aqueous suspension on to a glass wool plug in the bottom of a Pyrex glass tube with a 1 mm constriction at one end, and a 20 ml bulb reservoir at the other.

1.5 ml of fresh serum is passed slowly through the column which is then washed with 50 ml of water to remove protein, sugars etc. The amino acids are eluted with 20 ml of 10 % $NH_4OH$ which is collected in a 50 ml conical flask and evaporated off under reduced pressure in a vacuum desiccator containing NaOH pellets and fused

$CaCl_2$. The residue is dissolved in 5 ml of warm water and 4 ml pipetted into a small test tube, 4 in. × ½ in., which had been previously treated with Silicone Repelcote (Hopkins and Williams). The water is again evaporated off under reduced pressure over NaOH pellets.

In this way, an extract, equivalent to 1.2 ml of serum, can be concentrated over a small area in a test tube, from which it can be conveniently removed quantitatively with a small volume of liquid.

*Electrophoresis*. The blood extract is partitioned into groups of amino acids by electrophoresis in *N* acetic acid on Whatman 3 MM paper in the conventional type of horizontal apparatus (see *e.g.* MCDONALD[21], WIELAND AND FISCHER[22]).

The serum extract is transferred to a strip of Whatman 3 MM paper, 5 cm wide and 20 in. long, by a technique designed to obtain even distribution along the load line, and to prevent amino acids running along the edges of the paper. The dry residue is dissolved in 0.05 ml of warm water and evenly distributed over an area of paper 4 cm × 3 cm adjacent to the load line which is 2½ in. from the anode end of the strip. This is allowed to partially dry, and the tube rinsed out with a further 0.05 ml of water which is transferred to the paper in a similar fashion and evaporated by a cold air blast. The strip is clamped in the electrophoretic apparatus, and wetted evenly with *N* acetic acid from both ends, so that the advancing liquid fronts meet at the loading line. In this way the compounds within the loading rectangle are dissolved up, and distributed along the starting line. Electrophoresis is carried out for 2½–3 h at 1,350 V, with a current passing of 2½ mA/strip of paper 5 cm wide. At the end of this time the paper is dried *in situ* by a stream of cold air.

Early experiments demonstrated that to obtain reasonable separations it was essential to minimise electro-osmotic flow of buffer solution, and the customary wet anode has therefore been substituted by a 26 SWG platinum wire sandwiched between two perspex strips which are pressed together by elastic bands. When in operation the end of the paper is folded and nipped between the perspex strips, thus forcing the wire into the wet paper surface and ensuring a good electrical contact. Fig. 1 describes the paper and electrode layout.

After electrophoresis, the dried paper strips are immersed in acetone containing 0.1 % *o*-coumaric acid and dried at room temperature for 4–5 min, followed by heating in an oven at 100° for 1½–2 min. Exposure to ultra violet light from a Mazda MBW/U bulb produces yellow fluorescent bands of amino acid–coumaric acid complex, which can be easily outlined with pencil. The imino acids, proline and hydroxyproline, can also be visualised. Overheating and over exposure to ultra violet radiation should be avoided[19].

The most rapidly moving band containing ornithine, lysine, histidine and arginine, is sometimes rather diffuse and difficult to see, but it can be concentrated by ascending chromatography with water followed by drying and relocating by the ultra violet technique.

The outlined bands are excised, dropped into test tubes and extracted with 5 ml of water for at least 2 h with occasional shaking. Suitable aliquots of the solutions, usually 3–4 ml, are measured out into test tubes, 4 in. × ½ in., previously treated with Silicone Repelcote, and evaporated under suction in a desiccator over NaOH pellets.

The dry residue, concentrated over a very small area in the bottom of the tube, is dissolved in 0.02 ml of water, and applied as a thin streak[23], 2 cm long, to a sheet of Whatman No. 1 filter paper 22 ½ in. long by 8 in. wide, drying being hastened by a cold air blast. The test tube is washed out twice more with 0.02 ml of water.

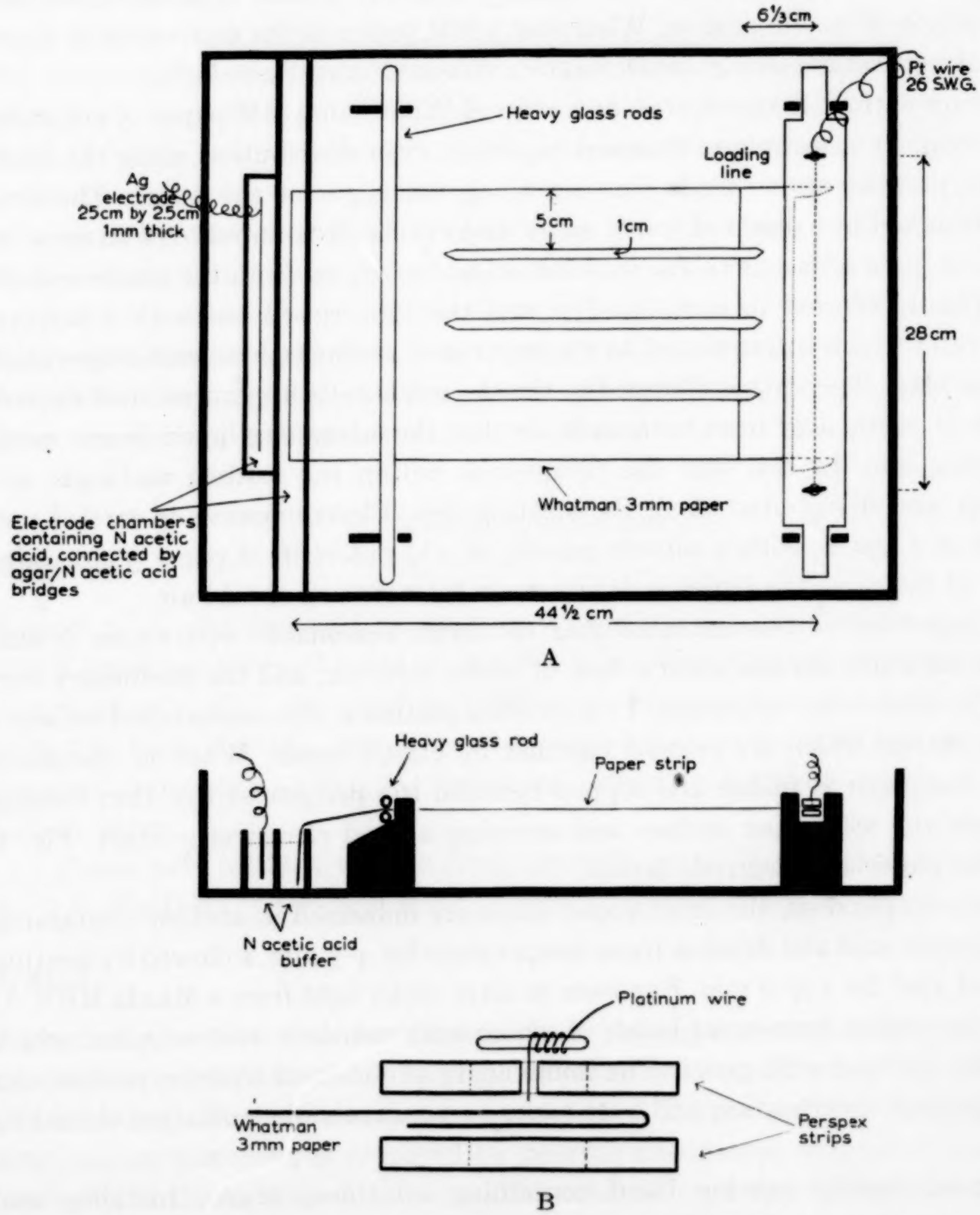
6⅓ cm
Pt wire
26 S.W.G.
Heavy glass rods
Loading line
Ag electrode 25cm by 2.5cm 1mm thick
5cm
1cm
28cm
Whatman 3mm paper
Electrode chambers containing N acetic acid, connected by agar/N acetic acid bridges
44 ½ cm
A
Heavy glass rod
Paper strip
N acetic acid buffer
Platinum wire
Whatman 3mm paper
Perspex strips
B

Fig. 1. Layout of the apparatus and paper for electrophoresis. (A) The apparatus, drawn to scale, is home built from perspex sheets. The thick paper is held at one end between two wide perspex strips (B) which are supported in two perspex holders, slotted vertically, and clamped at the other end between two heavy glass rods, resting on a perspex incline. This system holds the paper taut, and pools of electrolyte cannot accumulate. The liquid compartments are filled with $N$ acetic acid, and connected by two agar–$N$ acetic acid bridges not shown in plan. (B) An expanded plan of the platinum wire electrode.

The sheets are hung in a suitable glass tank, placed in the dark and the amino acids in the various groups are separated by descending chromatography, employing as solvent a mixture of *n*-butanol–ethanol–ethyl acetate–water and glacial acetic acid (see Table I). This solvent gives good separations, with very compact spots, especially if the amino acids are loaded on stripwise as described. The small amount of acetic acid present prevents "bearding" of valine, leucine and glutamine. The *o*-coumaric acid contamination in the extract has an $R_F$ value of 0.89, and therefore travels near the solvent front. Table I shows the overall separations achieved.

TABLE I

THE FREE AMINO ACIDS OF NORMAL HUMAN SERUM

Separations achieved by electrophoresis in *N* acetic acid followed by paper chromatography.

| *Electrophoresis* | *Time of development h* | *Paper chromatography* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Solvent:* | *butanol –* | *ethanol –* | *ethyl acetate –* | *water –* | *glacial acetic acid* |
| | | *Bands 1–5* | *60 ml* | *15 ml* | *10 ml* | *20 ml* | *2 ml* |
| | | *Band 6* | *40 ml* | *15 ml* | *10 ml* | *20 ml* | *20 ml* |
| Band 1 | 40 | Asp and Hypro | | | | | |
| Band 2 | 40 | CySH + $Cy_2S_2$, Asp-$NH_2$, Glu-$NH_2$ + Cit, Glu, Thr, Pro, Tyr, Met, Phe | | | | | |
| Band 3 | 24 | Ser, Val, Leu + Ileu | | | | | |
| Band 4 | 40 | Ala | | | | | |
| Band 5 | 40 | Gly | | | | | |
| Band 6 | 72–96 | Orn, Lys + His, Arg | | | | | |
| | | Increasing $R_F$ value → | | | | | |

At the appropriate times (Table I) the sheets are removed, dried in a stream of cold air, and then sprayed evenly with a solution consisting of 100 ml of 95 % ethanol plus 2 ml of lactic acid and 2 g of ninhydrin. The paper is air dried for 10 min at room temperature in a gentle air current, and then heated in an oven at 105° for 4 min. The coloured spots are cut out and dropped into test tubes containing 7.8 ml of 90 % ethanol and 0.2 ml of 1 % aqueous cadmium sulphate and allowed to stand at room temperature for 10–20 min. The pink solutions are then determined in the Spekker with the Ilford green filter (No. 604). Aliquots of a standard leucine solution (5 mg/ml), ranging from 2.5–50 $\mu$g are spotted on Whatman No. 1 filter paper, using an Agla micrometer syringe, and developed simultaneously with the paper chromatograms. The larger quantities of leucine are distributed in small spots, and under these conditions straight line graphs are obtained up to 50 $\mu$g of leucine. The individual amino acids can be calculated in terms of leucine, which is quite satisfactory for comparative work within the laboratory. The absolute values can be obtained by constructing standard curves of the various amino acids, or by establishing a table of factors required for converting leucine equivalents to the appropriate amino acid. The amino acids may also be estimated by extracting the blue spots with 70 % alcohol and rapidly reading against a yellow filter (Ilford No. 606) but the previously described technique is preferred as the colorimeter readings are increased by 60–70 %, and the pink colour is stable overnight.

This technique does not measure small quantities of hydroxyproline or proline, but it is possible to increase the sensitivity of the reaction with proline by a similar estimation in acid solution.

The yellow proline spot obtained by the routine technique of spraying and heating is excised and dropped into 8 ml of a solution of calcium chloride in acetic acid (2.5 ml of 20 % $CaCl_2$ + 97.5 ml of glacial acetic acid), then heated in a boiling waterbath for 5 min. The bright pink solution is rapidly cooled, and read with the Ilford 604 filter. The colours are stable for 15 min in daylight and at least 1 h in the dark. Reproducible straight line standard curves are obtained with 0-45 $\mu g$ of proline.

In the developmental stage of the method the separated amino acids were located by the fluorescence technique followed by excision and estimation by the excellent quantitative method of TROLL AND CANNAN[24]. The results, however, proved to be unreliable due to very high variable paper blanks. This background interference could be completely removed by treatment with $N/10$ NaOH[25] or by $N/20$ $Na_2CO_3$ but we have observed losses of amino acids by this system.

## RESULTS

Early experiments with mixtures of relatively few amino acids suggested that the speed of movement during electrophoresis altered slightly with the complexity of the mixture being separated, and location of the individual compounds was eventually achieved by running duplicate aliquots of serum extract, to one of which had been added a known amino acid.

Electrophoresis of serum extracts under the conditions described, normally gives six bands, five of which can be detected by the fluorescence method. Table I is a summary of the separations achieved by electrophoresis followed by paper chromatography. The acids in band 1, *i.e.* hydroxyproline, and aspartic acid, are usually present in concentrations too low to be visualised by fluorescence in the thick 3 MM paper. It is our practice to excise band 2 together with a 2 cm wide strip above it, thus combining bands 1 and 2.

This mixture contains thirteen amino acids, occurring in nine distinct spots, *i.e.* a diffuse double spot of cystine and cysteine, aspartic acid and asparagine, a deeply staining mixture of glutamine and citrulline, followed by the single amino acids glutamic acid, threonine, proline and tyrosine. Aminobutyric acid and methionine occasionally separate but are normally estimated together. Phenylalanine is the fastest moving compound in this group.

Band 3 contains serine, valine, and a fast moving double spot of leucine and isoleucine, which is not sufficiently separated for the estimation of each component. Occasionally this band also contains traces of glutamine and threonine from band 2, but these occur as individual spots before and after serine respectively.

Bands 4 and 5 contain no compounds other than alanine and glycine, and may conveniently be combined for paper chromatography. The basic amino acids which occur in band 6 move very slowly and require at least 72 h development for separation into three spots, *i.e.* ornithine, lysine and histidine as a poorly separated double spot, and arginine.

### *The colorimetric estimation of amino acids*

Under the standard conditions described, it was found that the slope of the leucine standard curves did not vary more than ± 3% over a period of several months. The slopes were invariably straight lines, as were the standard curves of twelve other amino acids studied. The absorption curves of the unstable blue colours produced

TABLE II

REPLICATION EXPERIMENTS

Estimations were carried out in quadruplicate on each of four separate specimens of pooled serum. Range of results, expressed as $\mu g$ of leucine.

| *Amino acid* | *Expt. 1* | *Expt. 2* | *Expt. 3* | *Expt. 4* |
|---|---|---|---|---|
| Asp-$NH_2$ | — | — | 0.8– 1.0 | — |
| Glu-$NH_2$ + Cit | 49.0–53.6 | 55.3–61.4 | 44.5–51.3 | 50.4–56.4 |
| Glu | 19.3–21.4 | 28.2–32.3 | 23.4–26.6 | — |
| Thr | 4.8– 5.8 | 5.8– 8.4 | 6.4– 7.6 | 5.4– 7.6 |
| Pro | 2.5– 2.8 | 0.7– 1.2 | 1.4– 1.9 | 0.3– 0.5 |
| Tyr | 2.9– 3.6 | 3.7– 4.0 | 2.6– 3.6 | — |
| Met | — | — | — | 1.9– 2.7 |
| Phe | 1.6– 1.9 | 0.5– 0.7 | 1.8– 1.9 | 0.3– 0.9 |
| Ser | 13.0–13.9 | 18.8–22.3 | 18.1–19.8 | 14.7–17.0 |
| Val | 22.2–27.2 | 34.8–38.4 | 31.2–32.0 | 30.4–35.0 |
| Leu | 14.5–16.6 | 18.5–21.7 | 16.9–17.9 | 17.6–19.0 |
| Ala | 46.0–48.2 | 37.7–40.6 | — | 41.2–46.0 |
| Gly | 15.9–18.8 | 14.9–16.1 | — | 17.0–17.6 |

TABLE III

RECOVERY OF AMINO ACIDS

The recovery of amino acids was tested by (a) estimating duplicate aliquots of serum to one of which had been added a known quantity of amino acid and (b) analysing "serum-like" synthetic mixtures of amino acids.

| *Amino acid* | *Amino acid added* $\mu g$ | *(a) Serum* % *recovery* | *(b) Synthetic mixtures* % *recovery* |
|---|---|---|---|
| Glu-$NH_2$ | 20 | 113 | |
| Cit | 25 | 102 | |
| Thr | 10 | 74 | 93 |
| Pro | 20 | 76 | 85 |
| Met | 5 | | 41 |
| Phe | 20 | 84 | 107 |
| Ser | 7 | 91 | 110 |
| Val | 20 | 90 | 93 |
| Leu | 20 | | 88 |
| Ileu | 25 | 79 | |
| Ala | 30 | 101 | 122 |
| Gly | 20 | | 124 |
| Orn | 9 | | 77 |
| Arg | 10 | 82 | 53 |
| Mean | | 89 | 83 |

by alcohol extraction gave rather broad maxima between 570–580 mμ, but the pink colours with added cadmium sulphate possessed sharp absorption curves with maxima between 510–515 mμ except that for phenylalanine which was at 500 mμ. The increased stability and sensitivity of the latter colour reaction is of considerable advantage in estimating the trace quantities of some amino acids present in serum.

### *The estimation of amino acids in synthetic mixtures and in serum*

The reproducibility of the method has been tested by running quadruplicate estimations on each of four separate pooled specimens of serum. The experimentally determined range of values for each amino acid, expressed as μg of leucine, are shown in Table II which clearly demonstrates that consistent replication may be obtained even below the 5 μg level.

Overall recovery experiments were then carried out in duplicate using either

TABLE IV

THE FREE AMINO ACIDS OF NORMAL FASTING HUMAN SERUM

The values given for each subject are the means for at least two specimens, drawn on different days.

| *Amino acid* | *Amino acid in serum, mg/100 ml* | | | | | | | *Mean (range)* |
|---|---|---|---|---|---|---|---|---|
| | *Subject C* | *Subject R* | *Subject G* | *Subject Q* | *Subject P* | *Subject B* | *Subject M* | |
| CySH + $Cy_2S_2$ | 0.6 | 1.7 | 0.8 | 1.2 | 0.7 | 1.2 | 1.1 | 1.0<br>(0.6– 1.7) |
| Asp + Asp-$NH_2$ | 1.4 | 1.7 | 3.0 | 1.9 | 2.7 | 2.3 | 2.7 | 2.2<br>(1.4– 3.0) |
| Glu-$NH_2$ + Cit | 8.7 | 11.5 | 10.6 | 8.9 | 8.9 | 7.6 | 7.8 | 9.1<br>(7.6–11.5) |
| Glu | 0.6 | 0.8 | 0.7 | 1.0 | 1.1 | 1.1 | 1.4 | 1.0<br>(0.6– 1.4) |
| Threo | 1.4 | 1.6 | 1.3 | 1.4 | 1.8 | 1.5 | 2.1 | 1.6<br>(1.3– 2.1) |
| Pro | 3.2 | 4.1 | 2.3 | 3.4 | 2.7 | 3.0 | 3.7 | 3.2<br>(2.3– 4.1) |
| Tyr | 1.3 | 2.3 | 1.3 | 2.0 | 1.6 | 1.4 | 1.4 | 1.6<br>(1.3– 2.3) |
| Met | 0.6 | 1.1 | 1.0 | 1.1 | 1.4 | 0.8 | 0.9 | 1.0<br>(0.6– 1.4) |
| Phe | 0.8 | 1.1 | 0.8 | 1.3 | 1.1 | 1.1 | 1.1 | 1.1<br>(0.8– 1.3) |
| Ser | 1.0 | 1.3 | 1.0 | 1.1 | 1.4 | 1.0 | 1.7 | 1.2<br>(0.9– 1.7) |
| Val | 2.5 | 3.1 | 2.5 | 3.2 | 2.8 | 2.7 | 2.6 | 2.6<br>(2.5– 3.2) |
| Leu + Ileu | 2.6 | 3.7 | 2.3 | 3.5 | 3.1 | 2.8 | 3.0 | 3.0<br>(2.3– 3.7) |
| Gly | 1.7 | 2.1 | 1.6 | 2.0 | 2.3 | 1.9 | 2.5 | 2.0<br>(1.6– 2.5) |
| Ala | 3.2 | 3.8 | 3.4 | 3.6 | 4.3 | 4.1 | 4.2 | 3.8<br>(3.2– 4.3) |
| Orn | 1.0 | 1.1 | 0.7 | 1.5 | 1.2 | 1.7 | 1.6 | 1.3<br>(0.7– 1.7) |
| Lys + His | 3.9 | 3.0 | 3.6 | 5.0 | 4.9 | 4.6 | 4.9 | 4.3<br>(3.0– 5.0) |
| Arg | 1.5 | 1.7 | 1.9 | 2.6 | 2.0 | 1.6 | 2.3 | 1.9<br>(1.5– 2.6) |

pure amino acids added to serum, or a mixture of amino acids in proportions resembling those in serum. The recoveries obtained, Table III, were usually between 80 % to 110 %, mean 86 %, and a subsequent quantitative study of the three main steps in the technique suggested that the major losses occurred prior to electrophoresis.

Initially the colorimetric readings from an analysis were calculated as leucine equivalents, but in later estimations, the specimens of serum were worked up in parallel with a synthetic serum-like mixture of amino acids, and the concentration of amino acid in the unknown calculated by simple proportion from the colorimetric readings. This procedure was considered to be justified by reason of the close replication obtainable and the straight line standard curves given by the different amino acids, and also has the advantage of internally correcting for any systematic analytical errors.

The serum levels of a small number of normal males were then investigated. Two fasting specimens of blood were taken from each subject, generally with an interval of two or more days, and the serum stored at —10° until required for analysis. The results of analysis showed that the individual pattern is remarkably constant over a small number of days.

The average pattern of values obtained for each of the seven subjects has been plotted in Table IV, which clearly shows that for these normal males there is little variation from the mean by the various amino acids. A similar consistency in values for each amino acid between individual subjects was shown by MOORE AND STEIN[2].

## TABLE V

### THE FREE AMINO ACIDS OF NORMAL SERUM OR PLASMA

The values obtained by the method described are compared, where possible, with values quoted in the literature.

| *Amino acid* | *Amino acid in plasma or serum, mg/100 ml* | | | | |
|---|---|---|---|---|---|
| | *Present work* (*a*) | MOORE AND STEIN[2] (*b*) | MCMENAMY *et al.*[26] (*c*) | BORDEN *et al.*[27] (*d*) | SALISBURY *et al.*[28] (*e*) |
| CySH + $Cy_2S_2$ | 1.0 | 1.2 | | | |
| Asp + Asp-$NH_2$ | 2.2 | 0.6 | | | |
| Glu-$NH_2$ + Cit | 9.1 | 8.8 | 6.6 | | |
| Glu | 1.0 | 0.7 | 0.3 | | 1.2 |
| Thr | 1.6 | 1.4 | 1.6 | 1.3 | 1.9 |
| Pro | 3.2 | 2.4 | 1.6 | 2.9 | 0.7 |
| Tyr | 1.6 | 1.0 | 1.3 | 1.0 | |
| Phe | 1.1 | 0.8 | 0.9 | 1.1 | 1.1 |
| Ser | 1.2 | 1.1 | | | 1.3 |
| Val | 2.6 | 2.9 | 2.0 | | 2.5 |
| Leu + Ileu | 3.0 | 2.6 | 1.8 | | 2.3 |
| Gly | 2.0 | 1.5 | | | 2.2 |
| Arg | 1.9 | 1.5 | 1.2 | 1.5 | 0.5 |

(a) Electrophoresis followed by paper chromatography.
(b) Chromatography on ion exchange columns.
(c) Paper chromatography.
(d) Microbiological technique.
(e) Microbiological technique.

Table V compares the average values obtained in this study with those published by other authors. There is a reasonable agreement on the whole, but for the large discrepancy between our value for aspartic acid plus asparagine, and that of MOORE AND STEIN[2]; this is, however, due to calculating the mixed spot in equivalents of aspartic acid which gives considerably less colour per molecule than asparagine in the modified ninhydrin reaction.

## SUMMARY

1. A method is decribed for the estimation of a number of free amino acids in serum.

2. The acids are extracted by Zeocarb 225H, and are then fractionated into six groups by electrophoresis at 1350 V in *N* acetic acid buffer. The various groups are located by a fluorescence technique employing *o*-coumaric acid in acetone, and are then separated into individual amino acids by descending paper chromatography. The solvent used is a mixture of butanol, ethanol, ethyl acetate, water and acetic acid. The final colorimetric estimation is made by spraying the paper with lactic acid and ninhydrin followed by elution with alcoholic cadmium sulphate. This technique produces a stable pink colour which is more sensitive than the normal ninhydrin blue.

3. Replication is close, with a mean recovery of about 86 %.

4. Fasting levels are given for seven males, and a comparison with other published values is made.

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# CHROMATOGRAPHIE SUR PAPIER DES DINITROPHÉNYLAMINOACIDES

(Addendum à l'article du *J. Chromatog.*, 2 (1959) 225)

GÉRARD BISERTE, JAMES W. HOLLEMAN, JANINE HOLLEMAN-DEHOVE
ET PIERRE SAUTIÈRE

*Laboratoire de Chimie Biologique de la Faculté de Médecine et de Pharmacie, Lille (France)*

(Reçu le 8 juin 1959)

## B. PRÉPARATION DES DINITROPHÉNYLAMINOACIDES

### *b. Cas particuliers de synthèse*

Dérivés de l'histidine: H. Zahn et H. Pfannmüller, *Biochem. Z.*, 330 (1958) 97.

α-DNP-histidine: H. Zahn et H. Pfannmüller, *Angew. Chem.*, 68 (1956) 40.

O-DNP-tyrosine: E. R. Fritze et H. Zahn, *Z. anal. Chem.*, 162 (1958) 414.

N,N'-Bis-DNP-mésolanthionine: H. Zahn et H. Pfannmüller, *Angew. Chem.*, 68 (1956) 41.

Réactions de condensation avec le 1,5-difluoro-2,4-dinitrobenzène: H. Zahn et J. Meienhofer, *Makromol. Chem.*, 26 (1958) 126 et 153.

## D. DINITROPHÉNYLATION D'UNE PROTÉINE

Dinitrophénylation de la laine à 60°: E. R. Fritze et H. Zahn, *Proc. Intern. Wool Textile Research Conf., Melbourne, Australia*, 1955, C-120.

## H. CHROMATOGRAPHIE DES DNP-AMINOACIDES HYDROSOLUBLES

Séparation de DNP-aminoacides hydrosolubles (S-DNP-Cys, O-DNP-Tyr, ε-DNP-Lys) des autres DNP-aminoacides et des acides aminés libres, par électrophorèse sur papier (Fig. 1). Electrophorèse à haut potentiel (6000 V, 6 à 8 mA), à basse tempé-

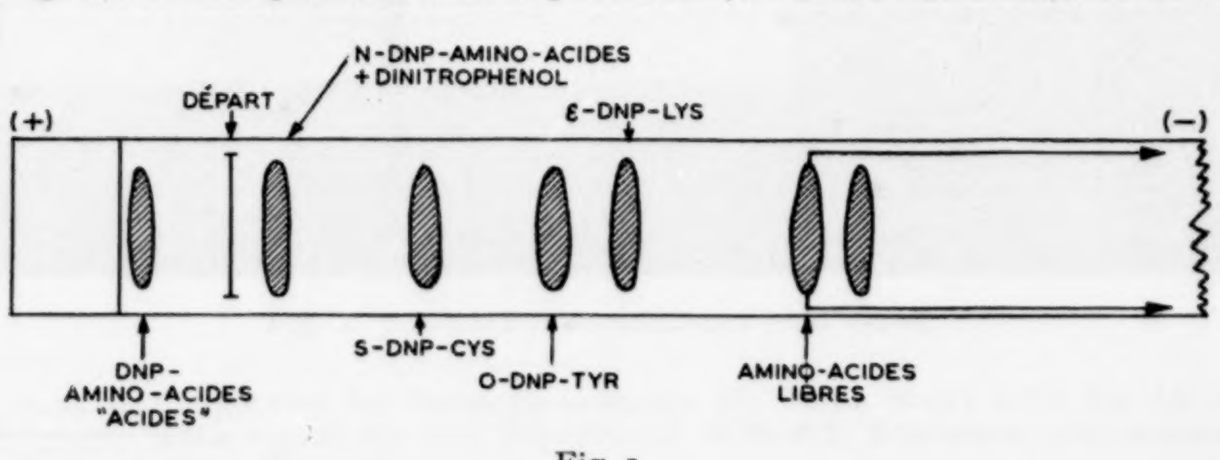

Fig. 1.

rature, pendant 180 min sur des feuilles de 80 × 14 cm placées dans un appareil de B. KICKHÖFEN ET O. WESTPHAL (*Z. Naturforsch.*, 7b (1952) 655). Tampon volatil acide formique–acide acétique de pH 1.93 (H. ZUBER, K. TRAUMANN ET H. ZAHN, *Proc. Intern. Wool Textile Research Conf., Melbourne, Australia*, 1955, C-127).

### J. CHROMATOGRAPHIE QUANTITATIVE DES DNP-AMINOACIDES

*Dosage de l'O-DNP-tyrosine et de l'ε-DNP-lysine*: E. R. FRITZE ET H. ZAHN, *Z. anal. Chem.*, 162 (1958) 414.

*Dosage de la S-DNP-cystéine*: A la température du laboratoire et à un pH inférieur à 5.5, le fluorodinitrobenzène réagit exclusivement avec le groupe SH de la cystéine. Après hydrolyse de la protéine, la S-DNP-cystéine séparée électrophorétiquement (voir plus haut) est dosée spectrophotométriquement à 330 mμ (H. ZUBER, K. TRAUMANN ET H. ZAHN, *Proc. Intern. Wool Textile Research Conf., Melbourne, Australia*, 1955, C-127).

### ERRATUM

Dans la Fig. 5, page 246, les positions de la DNP-leucine et la DNP-phénylalanine ont été inversées.

# Short Communications

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## An apparatus for the equilibration of columns prior to use in vapor phase chromatography*

In an effort to separate many diverse types of compounds by vapor phase chromatography, it became necessary to evaluate a large number of potentially useful column packing materials. The equilibration of the packed columns while connected to the chromatographic instrument** precluded its analytical use and occasionally fouled the conductivity cell. Hence, other means of conditioning the columns were sought. The usual practice of conditioning the columns independently (merely placing the columns in an insulated rack and heating electrically while nitrogen flow is maintained) was found to be unsatisfactory because of the high rate of convection cooling and the

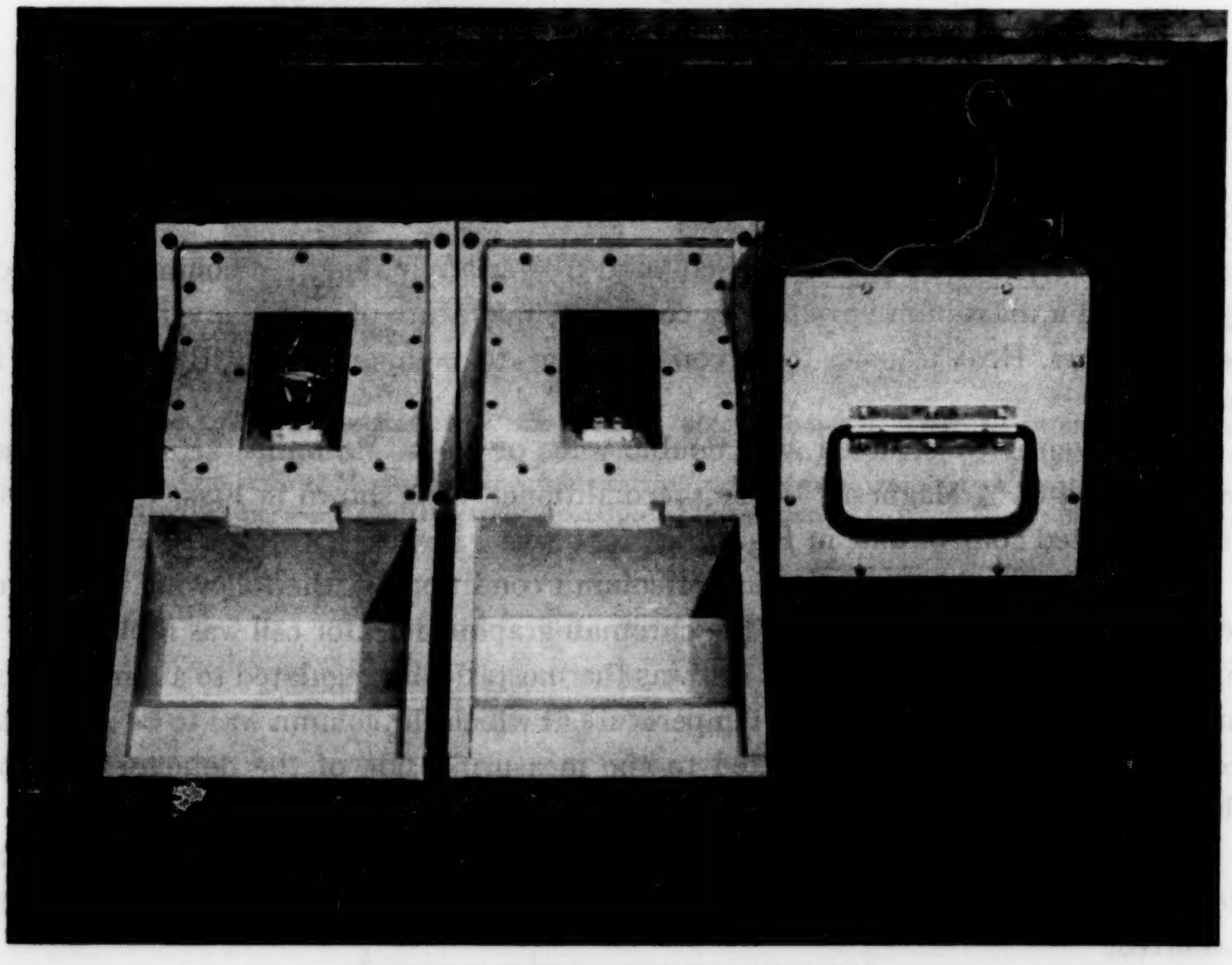

Fig. 1. 3-column preconditioner (end view).

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** Burrell Kromo-Tog: Model K-2.

presence of uneven heating. For these reasons, a device was designed specifically for the conditioning of columns. With a minimum of conversion it may also be used as a three-unit chromatographic instrument.

*Apparatus.* The preconditioner built in this laboratory is essentially a well insulated chamber with provision for the flow of heated gas through the column while it is held at some predetermined temperature. This particular unit is designed to service 3 Burrell hairpin type columns of any length up to 2.5 m with individual control of gas flow and heater voltage. It is capable of maintaining temperatures in excess of 400°, shows a maximum temperature variation along the column length of ± 0.5° and a maximum variation in column temperature of ± 1.0° over a period of several hours. Heat transfer from compartment to compartment within the unit is negligible.

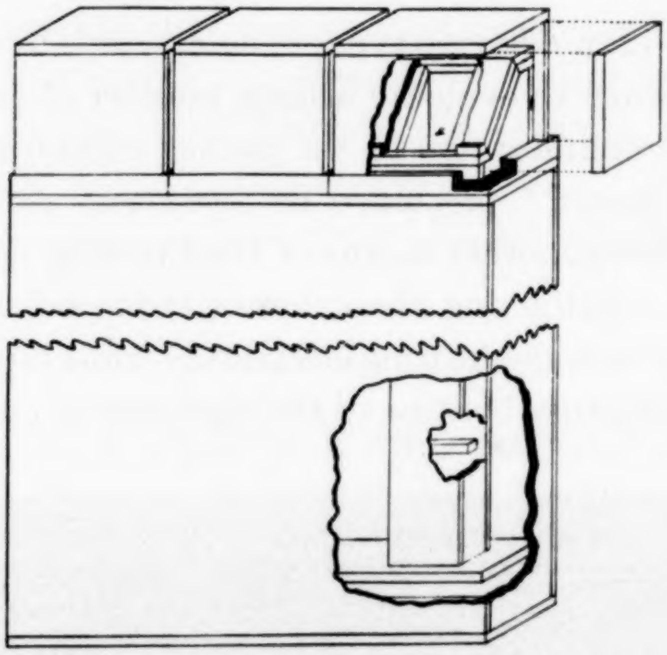

Fig. 2. 3-column preconditioner (cut-away view).

The oven is constructed with double walls of ½ in. Maronite* separated by at least 1 in. of 85% Magnesia**. The preconditioner*** is shown in Figs. 1 and 2 and the associated control unit in Fig. 3.

*Methods.* To obtain a criterion of column conditioning, the following procedure was set up: The reference side of the chromatographic detector cell was replaced by a fixed resistance, and the detector cell was thermostatically regulated to a temperature at least as high as the maximum temperature at which the column was to be used. The column in question was connected to the measuring side of the detector cell and flushed with inert gas at room temperature until a stable base line was obtained.

After attainment of a stable base line with the column at room temperature, the column temperature was gradually raised until upward deflection of the base line occurred.

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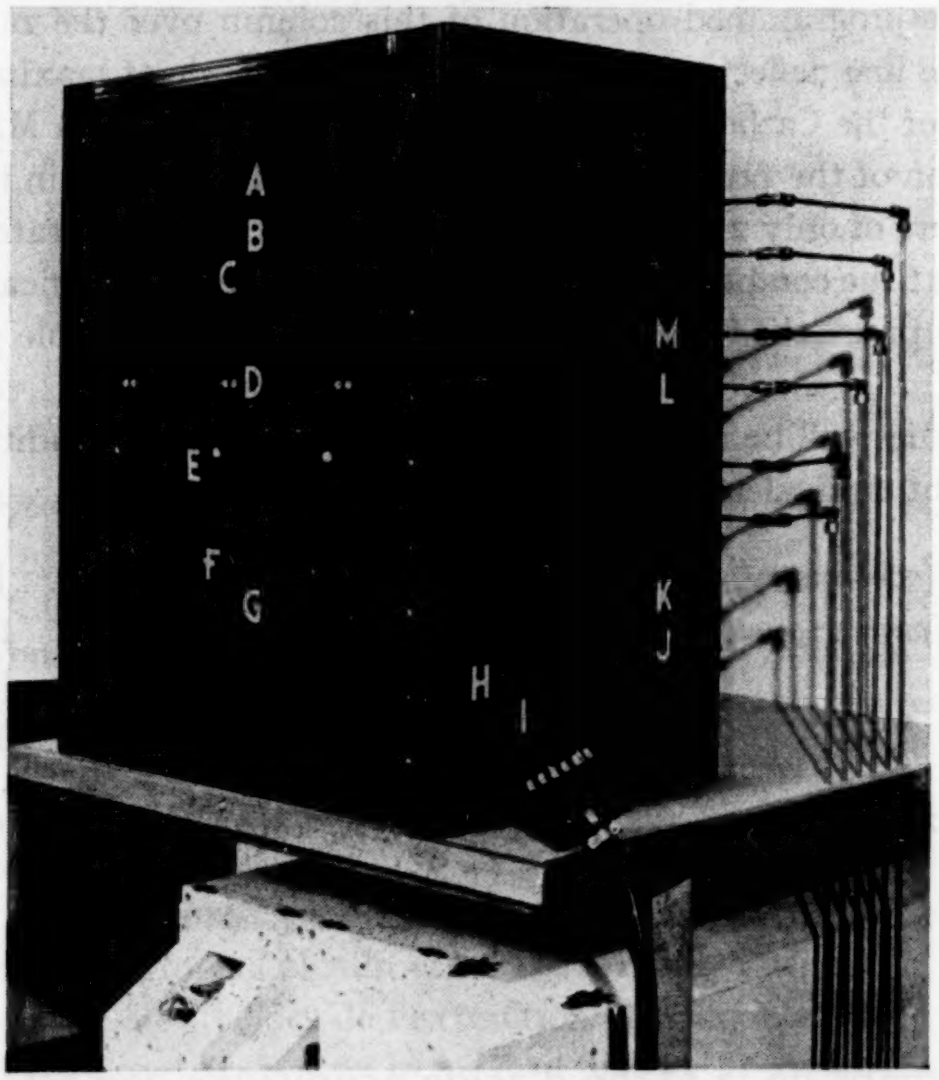
* Maronite, Johns Manville Co.: A sheet product composed of asbestos fiber and diatomaceous earth with an inorganic filler.

** 85% Magnesia, Johns Manville Co.: A coarse powder containing 15% asbestos fiber.

*** Blueprints can be made available on request.

The temperature at which the base line showed a deflection of 0.5 % full scale at maximum sensitivity was considered to be the maximum allowable operating temperature (MAOT) for each column after the particular conditioning applied.

*Results and discussion.* Fig. 4 indicates the manner in which conditioning time affected the MAOT for a pair of typical columns. The shape of the curve appears to be



.5%

Fig. 3. 3-column preconditioner control unit. A, flow meter; B, needle valve; C, input and output toggle valves; D, thermistor outlets; E, powerstat; F, switch, fuse and pilot light cluster; G, auxiliary powerstat outlet; H, cable connector, preconditioner to control unit; I, main power inlet; J, gas inlet, from tank; K, gas outlet, to atmosphere; L, gas stream, to oven No. 2; M, gas stream, from oven No. 2.

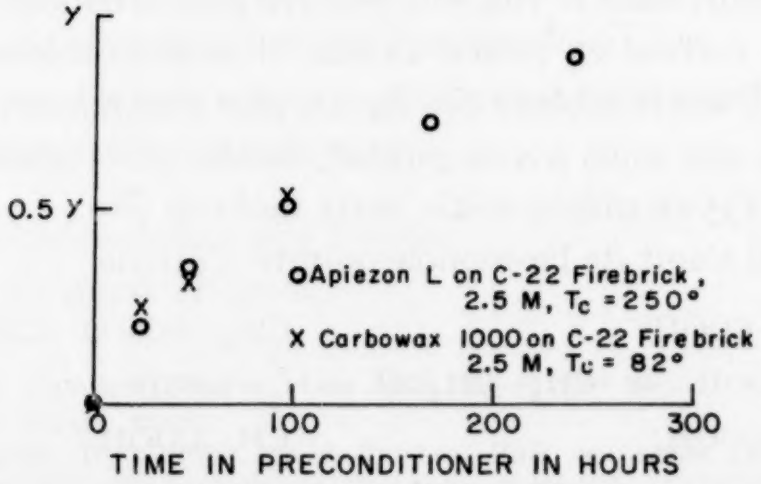


Fig. 4. Plot of $Y$ as a function of time for a constant preconditioner temperature where: $Y = \frac{T - 20}{T_c}$; $T$ = maximum allowable operating temperature, and $T_c$ = preconditioner temperature.

sufficiently regular to permit the prediction of required conditioning times on this basis.

The Apiezon L column was maintained at a temperature of 250° in the preconditioner until the desired MAOT of 245° was attained. For this column 4 days of conditioning per m column length were required. This conditioning time is far in excess of that usually considered adequate, and yet is absolutely essential for satisfactory temperature-programmed operation.

Temperature-programmed operation of this column over the range 0–240° has resulted in a base line deflection of less than 0.5 % full scale at maximum sensitivity.

In the case of the Carbowax 1000 column shown in Fig. 4, an MAOT of 45° was desired. Operation of the preconditioner at 82° allowed us to obtain this MAOT with a time expenditure of only 2 days per m column length. This illustrates the somewhat evident point that the conditioning time for a column may be significantly reduced by operation of the preconditioner at a temperature as far as possible in excess of the MAOT desired.

Further studies will be required to determine the effect of conditioning time and rate on the height equivalent theoretical plate.

*Department of Biochemistry, Baylor University College of Medicine, Houston, Tex. (U.S.A.)*

J. A. COOPER*
R. CANTER
F. L. ESTES
JOSEPH H. GAST

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* Public Health Service Research Fellow of the National Heart Institute.

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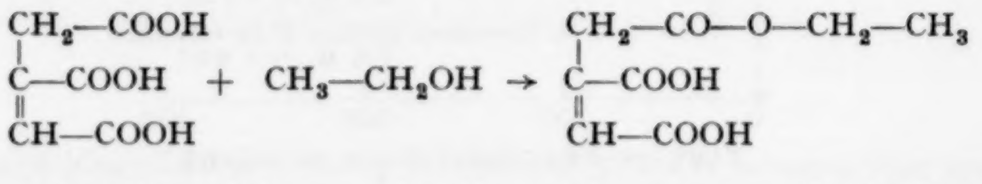
## Dédoublement de la tache d'acide aconitique par estérification

Dans les études chromatographiques d'acides organiques, il est une tache maintes fois signalée: JERMSTAD ET JENSEN[1], par exemple, l'ont trouvée parmi les acides organiques de l'Aconit et l'ont attribuée à quelque acide inconnu. Nous avons retrouvé cette tache dans l'Aconit, dans le Blé, etc. Son $R_F$ est nettement plus grand que celui de l'acide aconitique, surtout en milieu alcalin; il se situe même légèrement au delà de l'acide fumarique. Dans le tableau des $R_F$, ou plus exactement des $R_G$ (Glycolique), des acides organiques que nous avons publié[2], tandis que l'acide aconitique va à 20 en milieu alcalin et à 135 en milieu acide, cette tache se situe à 75 en milieu alcalin et 155 en milieu acide: il s'agit de l'α-monoaconitate d'éthyle



$$\begin{array}{l} CH_2{-}COOH \\ | \\ C{-}COOH \\ \| \\ CH{-}COOH \end{array} + CH_3{-}CH_2OH \rightarrow \begin{array}{l} CH_2{-}CO{-}O{-}CH_2{-}CH_3 \\ | \\ C{-}COOH \\ \| \\ CH{-}COOH \end{array}$$

Après extraction alcoolique faite sur des tissus où se trouve de l'acide aconitique, il apparaît régulièrement en assez grande abondance. L'alcool éthylique étant le

liquide d'extraction le plus classique, pas plus que Jermstad nous n'avons d'abord soupçonné que cette tache pouvait provenir d'un artefact. Nous avons isolé et fait cristalliser cet acide, nous l'avons analysé du point de vue cristallographique, spectrographique, etc.[3]. Comme il fournit de l'acide aconitique à l'hydrolyse, nous pensions à quelque dérivé naturel, mais, lorsque nous avons eu identifié l'aconitate d'éthyle, lorsque nous avons eu synthétisé ce produit, nous avons constaté qu'il se forme au cours de l'extraction alcoolique et qu'il suffit de faire l'extraction avec un solvant non-alcoolique pour que ce corps n'apparaisse pas.

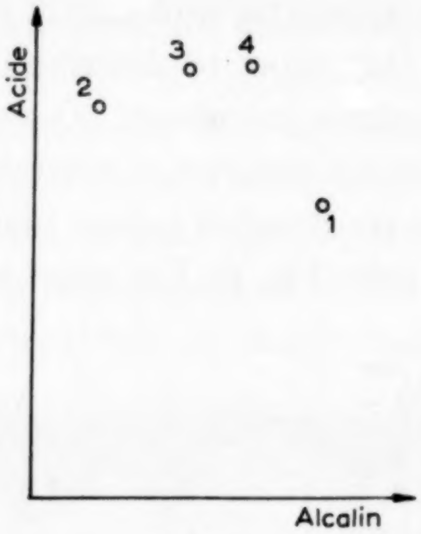


Fig. 1. (1) Acide glycolique. (2) Acide aconitique. (3) Aconitate de méthyle. (4) Aconitate d'éthyle.

Si l'on emploie l'alcool méthylique, il se forme, au lieu du monoaconitate d'éthyle, du monoaconitate de méthyle. Comme le laissait prévoir la différence des poids moléculaires, le $R_F$ de l'aconitate de méthyle est moins grand que celui de l'aconitate d'éthyle: il est de 50 en milieu alcalin et de 152 en milieu acide.

Les dangers pour les glucides de l'extraction alcoolique avaient déjà été signalés par Nottbohm et Mayer[4] qui ont pu isoler du Lupin de l'éthyl-α-D-galactoside formé pendant l'extraction alcoolique aux dépens du galactose; mais on n'avait rien signalé de tel pour les acides organiques. Il importe de savoir que l'acide aconitique est fragile, facilement estérifiable et susceptible de donner en chromatographie sur papier deux taches très nettes. Ces taches sont facilement identifiables, non seulement par leur $R_F$, mais par leurs réactions qui restent celles de l'acide aconitique, réaction de Godin, réaction à l'urée (formation d'acide citrazinique), etc.

Il est probable que d'autres acides organiques sont susceptibles de produire dans les mêmes conditions des esters éthyliques.

*Laboratoire de Physiologie Végétale de l'Institut Catholique de Toulouse (France)*

J. Carles
S. Lascombes

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[1] A. Jermstad et K. B. Jensen, *Pharm. Acta Helv.*, 25 (1950) 209.
[2] *cf. Chromatog. Data*, 1 (1958) xxiii.
[3] S. Lascombes, A. Lattes, R. Miquel et R. Pulou, *Bull. soc. chim. biol.*, sous presse.
[4] E. Nottbohm et F. Mayer, *Vorratspflege u. Lebensmittelforsch.*, 1 (1938) 254.

Reçu le 8 juin 1959

# Investigations of greenheart alkaloids by paper electrophoresis

Some applications of electrophoresis to the separations of alkaloids have been reviewed by LEDERER[1]. The limited use of this technique in alkaloid chemistry can be partly attributed to lack of suitable apparatus and partly to the lack of suitable current sources. It is the purpose of this paper to describe electrophoresis experiments on alkaloids from the British Guiana greenheart (*Ocotea rodiaei*), using an ordinary glass chromatography tank. The construction of a suitable D.C. supply is also described.

*Apparatus.* The apparatus consisted of a glass tank 30 × 35 × 45 cm fitted with a glass cover having a central hole (Fig. 1). The paper was held in the vertical position by means of a glass frame. The electrodes consisted of horizontally placed platinum wires sealed into glass tubing with holes. The platinum wires were connected to the external copper leads through mercury contacts. The copper leads were lacquered to prevent corrosion. A simple D.C. source is shown in Fig. 2. This circuit using the stabilising valves $V_2$ and $V_3$ supplies a constant current with slightly varying resistive load of the paper, which may be due to evaporation taking place.

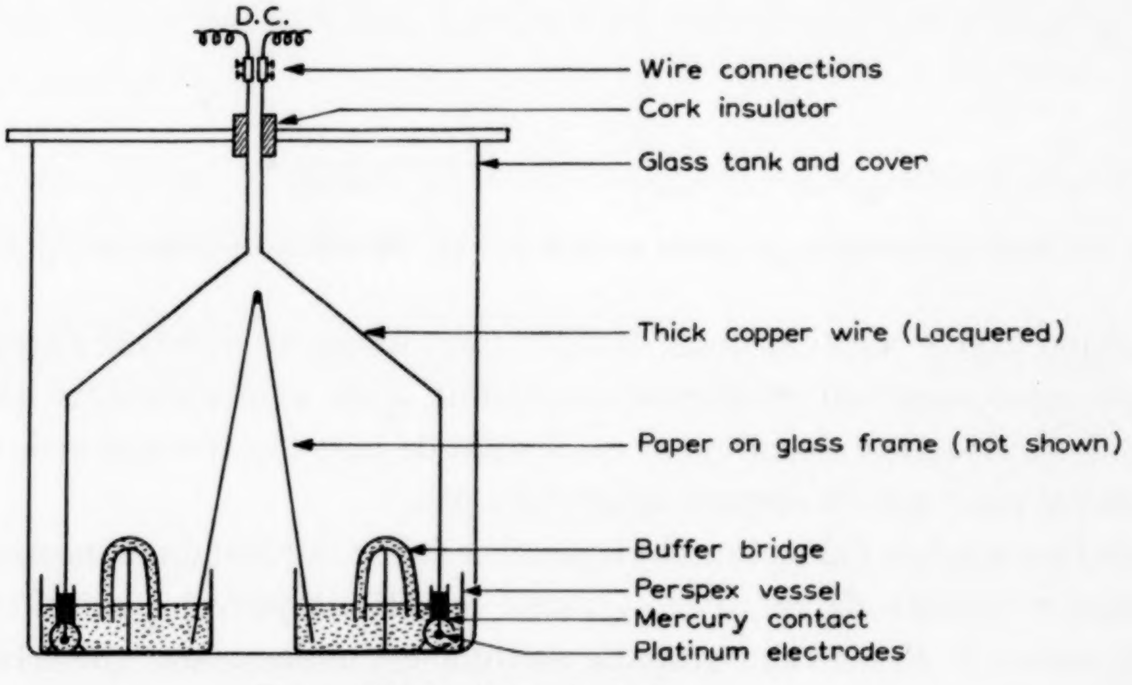
D.C.
Wire connections
Cork insulator
Glass tank and cover
Thick copper wire (Lacquered)
Paper on glass frame (not shown)
Buffer bridge
Perspex vessel
Mercury contact
Platinum electrodes

Fig. 1. Electrophoresis tank.

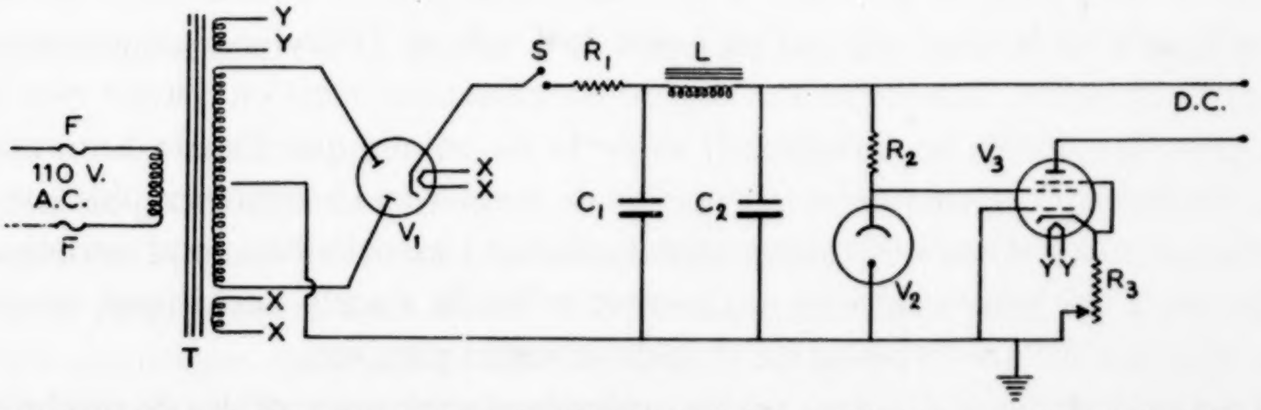
Y
Y
S
R1
L
D.C.
F
110 V.
A.C.
F
X
X
V1
C1
C2
R2
V3
V2
YY
R3
X
X
T

Fig. 2. Constant current D.C. circuit. $C_1$: 16 mfd. electrolytic 500 V; $C_2$: 32 mfd. electrolytic 500 V $R_1$: 100 $\Omega$, 1 W; $R_2$: 133 k$\Omega$, 1 W; $R_3$: 5 k$\Omega$ potentiometer 1 W; S: Switch; T: Transformer 250-0-250–60 mA; $V_1$: 5U4G; $V_2$: VR 150; $V_3$: 6B.W.6; L: 10 Henry choke, 300 $\Omega$, 40 mA F: 1 A fuses.

*Electrophoresis run.* Experiments were done in a temperature-controlled room ($T = 23 \pm 2°$). Whatman No. 1 paper sheets 53 × 23 cm were spotted near the apex with approximately 500 $\mu$g of the alkaloid mixture or 50 $\mu$g of single components. The buffer used consisted of a mixture of sodium acetate–acetic acid, pH 4.4, ionic strength 0.133. The paper was first wet with the buffer solution and after a short period for draining, the current was switched on and adjusted to 7 mA (*ca.* 200 V), with the variable resistance $R_3$. After 12–16 h the paper was removed and dried. The alkaloid spots were detected by dipping the paper in a saturated solution of iodine dissolved in petroleum ether. This was found to be a more satisfactory method of application than the iodine vapour detection method used by MUNIER AND MACHEBOEUF[2]. The alkaloids showed up as yellow or brown spots on a faint yellow or white back ground.

*Results.* The results of a typical run are shown in Fig. 3. The components of the mixture were only partially resolved. The similar electrophoretic behaviour of Base II and Base III isolated by counter-current distribution techniques was not surprising. Other chemical evidence indicated them to be very similar bases. Detailed structural investigations will be published elsewhere.

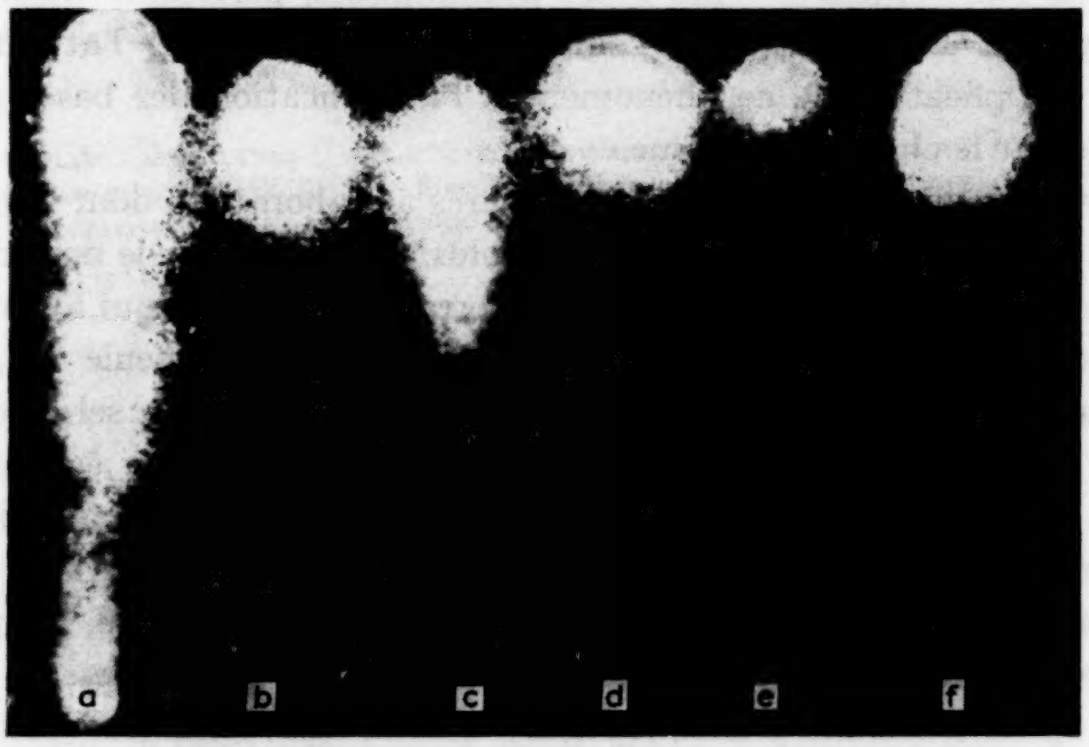

Fig. 3. a = Mixture of Greenheart alkaloids; b = Base III; c = Base III and IV; d = Base II. e = *d*-Tubocurarine chloride; f = *l*-Curine hydrochloride.

Two very closely related bisbenzyl isoquinoline alkaloids *l*-curine and *d*-tubocurarine chloride which were used as reference compounds also show similar electrophoretic behaviour.

*Chemistry Department, University College of the West Indies,* A. W. SANGSTER
*Jamaica (West Indies)*

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[1] M. LEDERER, *Introduction to Paper Electrophoresis and Related Methods*, Elsevier, Amsterdam, 1955, p. 92.

[2] R. MUNIER AND M. MACHEBOEUF, *Compt. rend.*, 230 (1950) 1177.

Received June 8th, 1959

# Disparition de l'uracil et de la thymine sur le chromatogramme par irradiation avec des rayons ultraviolets

D'après les travaux consacrés à la photodégradation des acides nucléiques, des nucléotides et des nucléosides, la photolyse de certaines matières est réalisée par les rayons ultraviolets (UV)[1,2,3]. Quant aux bases, on sait, par exemple, que la thymine en solution aqueuse est dégradée par irradiation, avec formation d'urée et d'acide pyruvique comme produits principaux[4]. En étudiant la photolyse des bases déposées sur du papier filtre, nous avons observé que cette photolyse est accompagnée d'une diminution de l'absorption UV des bases pyrimidiques, particulièrement de l'uracil et de la thymine. Le but de cette communication est d'attirer l'attention sur les possibilités d'application de ce phénomène à l'identification des bases puriques et pyrimidiques sur le chromatogramme.

Nous avons utilisé des échantillons préparés au laboratoire dont la pureté a été vérifiée par chromatographie sur papier. De plus, pour chacune de nos préparations, nous avons vérifié l'accord des spectres d'absorption avec ceux qui avaient déjà été étudiés précédemment[5]. Parmi les préparations employées, seule la guanine en raison de sa mauvaise solubilité, a été introduite sous forme de sel chlorhydrique, tandis que les autres ont été utilisées sous forme libre. Chaque échantillon a été employé en solution aqueuse à la concentration de 15 m$M$. Les irradiations ont été effectuées sur les composés déposés sur du papier filtre (Toyo No. 51-A) à la température ordinaire, en présence d'air, à 30 mm de distance, sans filtre, au moyen de la lampe germicide commerciale à vapeur de mercure à basse pression "National, GY-176·G, 15 W" (Matsushita Electric Ind. Co. Ltd., Osaka) qui émet principalement à 253.7 m$\mu$. La révélation des substances a été réalisée au moyen de la même source avec un filtre (253.7 m$\mu$) qui nous a été généreusement donné par le Laboratoire du Dr. IWASE à l'Institut de Recherches scientifiques, Tokyo.

Nous avons d'abord examiné la possibilité de diminution ou disparition des taches de quelques échantillons par cette technique. Dans ce but, on dépose 10 $\mu$l de solution sur la feuille de papier en taches de 7 à 9 mm de diamètre qui renferment donc 0.15 $\mu M$ d'échantillons. Après séchage à la température ordinaire, la feuille est irradiée comme indiqué ci-dessus. La Fig. 1(a) qui montre un exemple typique de cette expérience, permet de se rendre compte de la diminution de l'absorption UV au cours de l'irradiation. On peut voir que l'absorption de l'uracil et de la thymine disparaît pratiquement après 7–9 heures d'irradiation, tandis qu'il n'apparaît pas de différences notables chez les bases puriques. De même, on peut voir que l'absorption de la cytosine diminue très lentement. En comparant ces résultats à ceux qui ont été obtenus par irradiation à travers un filtre en verre de 1 mm d'épaisseur, on peut déceler un effet protecteur du verre ou mettre en évidence une photosensibilité des bases pyrimidiques envers les rayons UV (Fig. 1(b)). Nous avons examiné ensuite les possibilités d'application de ce phénomène à l'identification des bases sur le chromatogramme. A cet effet, nous avons déposé sur la ligne de départ une partie aliquote contenant la même quantité d'échantillon que dans l'expérience décrite ci-dessus. Les taches obtenues avaient environ 6 mm de diamètre. Après séchage, la feuille a été irradiée pendant 10 heures. Le chromatogramme a été développé selon la technique ascendante unidimensionelle par le solvant de WYATT[6]. Le papier a été alors retiré et abandonné à la température du laboratoire, à une lumière diffuse. Les résultats ainsi obtenus ont été plus nets pour l'uracil et la thymine, dont les taches n'apparaissent point. Il faut signaler cependant, que, dans les expériences avec le mélange des bases, il faut un temps d'irradiation plus long pour diminuer l'absorption des deux taches (Fig. 2(a)). Par contre, les deux taches sur le chromatogramme disparaissent après 6 heures d'irradiation (Fig. 2(b)). Lorsqu'on se sert de butanol–ammoniaque[7] comme solvant, on obtient à peu près le même résultat.

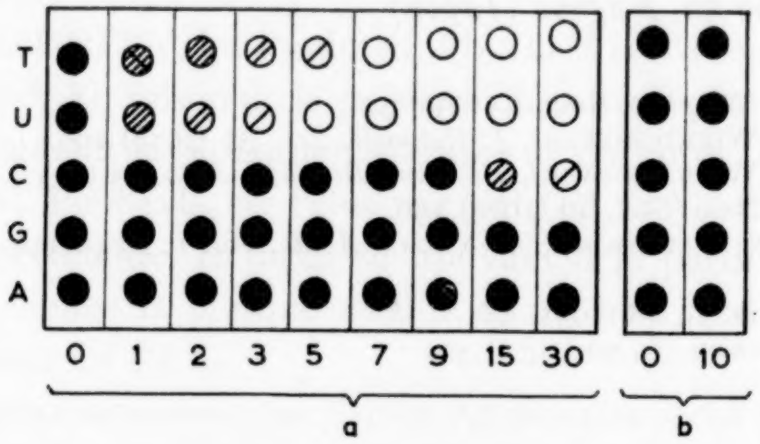

Fig. 1. Modification de l'absorption UV des bases déposées sur le papier filtre en fonction du temps d'irradiation par rayonnement UV (a) sans filtre ou (b) à travers un filtre en verre. Les chiffres montrent la durée d'irradiation en heures. A, adénine; G, guanine; C, cytosine; U, uracil; T, thymine.

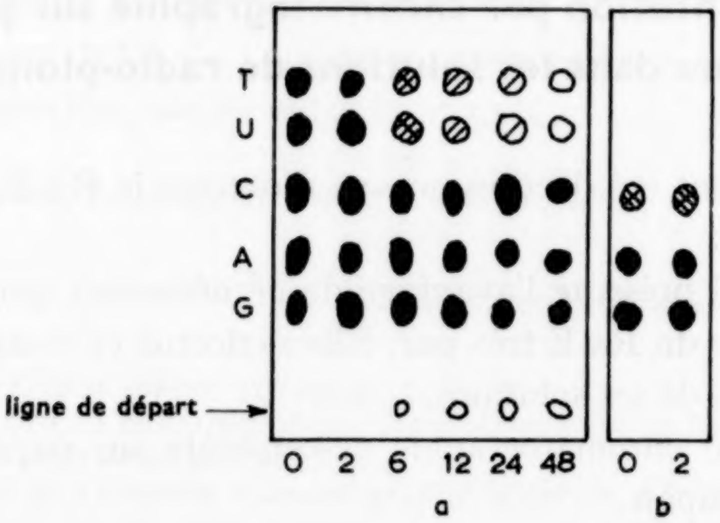

Fig. 2. Chromatogramme du mélange synthétique dans l'isopropanol/acide chlorhydrique/eau, mettant en évidence la diminution ou disparition des taches des bases pyrimidiques, en particulier de l'uracil et de la thymine. (a) Les chiffres indiquent la durée d'irradiation en heures avant développement. (b) La partie gauche du chromatogramme correspond au temps zéro et à 2 heures d'irradiation dans la Fig. 2(a), qui a été révélée après 6 heures d'irradiation.

Nous nous limitons ici à remarquer que la technique proposée peut rendre service à l'identification des bases à cause de la photosensibilité de l'uracil et de la thymine envers les rayons UV.

Nous remercions M. le Professeur F. EGAMI de l'Université de Tokyo qui nous a aimablement guidé et encouragé durant ces travaux.

*Laboratoire de Chimie, Université municipale de Nagoya,*
*Mizuho-ku, Nagoya (Japon)*
HIROSHI ISHIHARA

1 M. ERRERA, *Biochim. Biophys. Acta*, 8 (1952) 30.
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7 W. S. MACNUTT, *Biochem. J.*, 50 (1952) 384.

Reçu le 8 juin 1959

*J. Chromatog.*, 3 (1960) 94–96

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## Extraction et purification par chromatographie sur papier de Ra E contenu dans les solutions de radio-plomb

De nombreuses méthodes ont été décrites en vue d'obtenir le Ra E pur à partir de solutions de radio-plomb[1, 2].

La méthode décrite ici présente l'avantage de ne nécessiter que des opérations simples et permet d'obtenir du Ra E très pur. Elle s'effectue en trois stades:

1. Extraction du Ra E de ses solutions.
2. Purification par: (a) chromatographie descendante sur papier; (b) chromatographie ascendante sur papier.

### 1. *Extraction du Ra E*

La solution que nous avons utilisée était une solution chlorhydrique 0.3 *N* contenant environ 10 mC de Ra D, ses descendants (Ra E, Po ou Ra F, Ra G) et quelques dizaines de mg de chlorure de plomb. Elle provenait de résidus de minerais d'uranium traités en vue de l'extraction du radium.

Dans la solution chauffée au bain-marie aux environs de 60° est immergée une lame de nickel d'environ 1 cm$^2$ animée d'un mouvement rotatif (environ 1 tour/sec). Sur cette lame se déposent le Ra E et le Po par dépôt spontané, le Ra D et le Pb par adsorption. Après quelques minutes, la lame de nickel est extraite de la solution, lavée à l'eau distillée, puis attaquée superficiellement par quelques gouttes d'acide nitrique concentré. La solution nitrique ainsi obtenue qui contient Ni, Ra E, Po et très peu de Ra D + Pb est évaporée, reprise par HCl concentré, évaporée de nouveau; le résidu est finalement dissous par une goutte de HCl *N* (solution A).

### 2. *Purification*

La purification s'opère en deux stades:

(*a*) *Elimination du Ni par chromatographie descendante sur papier.* Une bande de papier Whatman No. 1 d'environ 2 cm de large et 40 cm de long, est préalablement lavée avec du butanol saturé de HCl *N*. A environ 3 cm d'une des extrémités de la bande, est alors déposée la solution A.

En milieu butanol–HCl *N*, les $R_F$ du Ni, Pb, Ra E et Po sont respectivement de 0.08, 0.15, 0.5 et 0.8[3,4]. En raison de la présence d'une trop grande quantité de Ni, il n'est pas possible d'obtenir la séparation sélective de ces corps. Il est donc indispensable d'extraire ce Ni. Pour cela, on l'élue par $NH_4OH$ 10 *N*, suivant la méthode de chromatographie descendante, la tache se trouvant à la partie inférieure. Lors de cette opération, on élimine la presque totalité du Ni sans modifier sensiblement le comportement ultérieur du Ra E.

(*b*) *Chromatographie ascendante sur papier.* Après avoir séché le papier, on procède à une chromatographie ascendante en milieu butanol–HCl *N* pendant une nuit. La partie de la bande Whatman contenant le Ra E, s'étalant depuis le $R_F$ 0.45 jusqu'au $R_F$ 0.55, est découpée et éluée avec de l'HCl *N*.

A l'aide de cette méthode, nous avons ainsi préparé des solutions de Ra E ne laissant pas de dépôt visible après évaporation.

Le rendement total de l'opération, compte tenu de la décroissance du Ra E pendant la purification, oscille entre 85 et 95 %.

*Institut du Radium, Laboratoire Curie, Paris* (*France*)

P. Conte
R. Muxart
avec la collaboration technique de H. Arapaki

[1] M. Haissinsky, *J. chim. phys.*, 31 (1934) 43.
[2] G. Bouissières et C. Ferradini, *Anal. Chim. Acta*, 4 (1950) 610.
[3] M. Lederer, *Thèse de doctorat*, Paris, 1954.
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Reçu le 25 mai 1959

*J. Chromatog.*, 3 (1960) 96–97

## A modified Ehrlich benzaldehyde reagent for detection of indoles on paper chromatograms*

Many modifications of the Ehrlich benzaldehyde reagent (EBR) for the detection of indoles on paper chromatograms are in current use[1,2]. Most of these suffer from the same limitations, namely (1) the time required for color development and (2) rapid fading of color.

We have found that these limitations can be markedly reduced by using the

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* This investigation was supported, in part, by Research Grant No. M-1015 (C2) from the National Institute of Mental Health, National Institutes of Health, U. S. Public Health Service.

Ehrlich benzaldehyde reagent followed by a nitrite spray. Our procedure is as follows:

1. The chromatogram is first sprayed with a 2% solution (w/v) of *p*-dimethylaminobenzaldehyde dissolved in conc. hydrochloric acid (12.1 *N*).
2. After an interval of 2–3 min, the chromatogram is sprayed with a 1% solution (w/v) of $NaNO_2$ in distilled $H_2O$.

Immediately, after the nitrite spray, indole compounds generally appear as deep blue spots with the exception of indican which appears as an orange brown spot. Urea appears as a large deep yellow spot. Colors developed with this reagent often persist for a month or more with little fading.

*Laboratory of Research Biochemistry, U.S. Veterans Administration Hospital, Coatesville, Pa.*
*and*
*Department of Psychiatry, School of Medicine, University of Pennsylvania, Philadelphia, Pa.* (*U.S.A.*)

HERBERT SPRINCE

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[1] C. E. DALGLIESH, *J. Clin. Pathol.*, 8 (1955) 73.
[2] J. B. JEPSON, in I. SMITH, *Chromatographic Techniques*, Interscience, New York–London, 1958.

Received May 21st, 1959

## BOOK REVIEWS

*Protides of the Biological Fluids*, Proceedings of the Fifth Colloquium, Bruges, 1957. Edited by Dr. H. PEETERS, published by Elsevier Publ. Co., Amsterdam, 1958, 260 pages, price 45 s.

The importance of electrophoretical techniques in the analysis of fluids containing proteins, *e.g.*, in the analytical study of biological fluids, both normal and pathological, can be well estimated from this book, edited by Dr. H. PEETERS, which contains all the papers presented at the Fifth Colloquium on Biological Fluids held in Bruges in 1957.

Some of these communications have already been published in *Clinica Chimica Acta*, but in this book all the communications to the colloquium have been collected in one volume. The volume will therefore be extremely useful for all those who in their daily work are confronted with the task of making a diagnosis with the help of chemical analysis.

The book contains 40 papers, some of which deal with general topics, while others are concerned with new methods and techniques of analysis. Of the contributors Prof. A. TISELIUS, Dr. K. HANNIG and Dr. C. WUNDERLY may be mentioned, who are well known for the part they have played in the development of these techniques.

The Round Table Conference, held at the colloquium, on the standardization of electrophoretical methods in view of their clinical applications, is also included. The average standard of the communications is very high and we are indebted to the Editor, Dr. H. PEETERS, for the organization of the colloquium and for this excellent book.

G. B. MARINI-BETTÒLO (Rome)

*J. Chromatog.*, 3 (1960) 98–99

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*Fortschritte der Verfahrenstechnik 1956/57*, Vol. 3, edited by H. MIESSNER AND U. GRIGULL (Farbenfabriken Bayer AG, Leverkusen), published by Verlag Chemie GMBH, Weinheim/Bergstr., 1958, 977 pages, price DM 66.—.

Like its predecessors (Vol. 1: 1952/53 and Vol. 2: 1954/55), Volume 3 of this series has attempted to review chemical industrial processes for industrial as well as academic chemists. The chromatographer will find numerous chapters of interest; however, one chapter on adsorption and ion exchange (Chapter 17 by K. BRATZLER) will be found particularly useful. In its 34 pages industrial adsorption and ion exchange processes are reviewed very concisely. The industrial manufacture of adsorbents is reviewed in three pages and like the rest deals with many processes which formerly could only be found in the patent literature. The various attempts at continuous adsorption, ion exchange and methods resembling chromatography are well discussed.

The whole chapter contains much stimulating material for the laboratory chemist. Amongst the 283 references one notes many patents and much German literature. The English literature is used often only as illustrative examples and is by no means complete.

As each chapter has its own bibliography the extensive subject and author indexes are very welcome. The book is well printed and no serious errors were noted.

M. LEDERER (Arcueil)

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*Progress in Nuclear Energy, Analytical Chemistry*, Vol. I (edited Proceedings of the Second International Conference on the Peaceful Uses of Atomic Energy, Geneva 1958). Edited by M. T. KELLEY, published by Pergamon Press, London, 1959, 372 pages, published price £ 5.5.0 net.

The papers are arranged in chapters as follows: 1. Reactor applications; 2. Activation analysis; 3. Spectrographic techniques; 4. Industrial applications; 5. Health physics. Chromatographic methods are mentioned in several of the review papers: on page 13 the separation of U and Th from large amounts of Bi by ion exchange, on pages 36–38

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a review of chromatographic and solvent extraction methods for the separation of Th, on page 61 an ion-exchange separation of Nd and Pr from Pu-rich fast reactor fuel, on pages 78–83 a review on the separation of U by chromatography and solvent extraction.

Unfortunately there is no introduction to this volume to explain such matters as what happened to the papers that were not selected, whether a second volume exists or is in preparation or whether a complete edition of the proceedings has been published elsewhere.

The reviewer naturally regrets that none of the numerous original papers dealing with chromatography which were presented at the conference (for example by T. SCHÖNFELD, K. A. KRAUS, R. G. DE CARVALHO) have been selected by the editors. There is a subject index of 4½ pages, then a list of some papers relating to analytical chemistry which were read in other sessions (Basic Chemistry in Nuclear Energy) and finally a list of the contents of the previous volumes that have appeared in this series.

M. LEDERER (Arcueil)

*J. Chromatog.*, 3 (1960) 99–100

## Announcement

An international Symposium will be held in Milan, Italy, on June 2–3–4 1960 for the purpose of reviewing the present status of biological and clinical research on "drugs affecting lipid metabolism". The agenda will include four main subjects:

1. New data on cholesterol and lipid metabolism (biosynthesis, absorption site, catabolism, excretion, abnormal pathways).
2. Experimental methods for the evaluation of drugs affecting cholesterol and lipid metabolism (new analytical methods and pharmacological tests).
3. Drugs affecting cholesterol and lipid metabolism in relation to the prevention and treatment of experimental atherosclerosis.
4. Clinical methods and therapeutical significance of drugs affecting cholesterol and lipid metabolism.

The Symposium, sponsored by the Institute of Pharmacology of the University of Milan, will be under the Chairmanship of Prof. E. TRABUCCHI.

Information on participation and presentation of papers can be obtained from Prof. S. GARATTINI, c/o Institute of Pharmacology, Via A. del Sarto 21, Milan, Italy, until March 1st, 1960.

The official languages are Italian and English. Simultaneous translation will be available.

The registration fee is US $ 10.

Further announcements will give information about the detailed program.

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# THE CHROMATOGRAPHY OF POLYCYCLIC AROMATIC HYDROCARBONS ON PARTIALLY ACETYLATED CELLULOSE

## PART II

T. M. SPOTSWOOD

*Department of Organic Chemistry, University of Adelaide, S.A. (Australia)*

(Received April 14th, 1959)

In an earlier publication[1], the application of reversed-phase chromatography on partially acetylated paper to the separation and identification of polycyclic aromatic hydrocarbons was described. The techniques previously reported have now been supplemented by alternative methods of locating compounds on the developed chromatograms, by the isolation of the compounds in crystalline form, and by the introduction of an improved technique of acetylation which has been found to produce papers of increased resolving power and better mechanical properties.

The separations obtained on partially acetylated paper have been reproduced on a larger scale by the use of columns of partially acetylated cellulose powder, using similar solvent systems to those used with partially acetylated paper. The resolutions obtained are comparable to those obtained on partially acetylated paper and in the case of compounds of relatively low $R_F$ values, are sometimes superior. The technique is of particular value in the separation of the higher polycyclic aromatic hydrocarbons, which are eluted almost simultaneously from an alumina column but have widely differing $R_F$ values. For example, a mixture of 3:4-benzopyrene, perylene, 3:4-benzofluoranthene, 10:11-benzofluoranthene, 11:12-benzofluoranthene, 1:12-benzoperylene and 2:3:10:11-dibenzoperylene can be resolved into constituents by this method without appreciable overlap of fractions.

### CHROMATOGRAPHY ON ACETYLATED PAPER

The technique of acetylation described previously[1] required the use of purified reagents in order to obtain acetylated papers of approximately reproducible properties. The amount of sulphuric acid used as catalyst was small compared with the total weight of the acetylating mixture, making the degree of acetylation obtained strongly dependent on relatively minor variations in the purity of the acetylating reagents. However, it has been found that chromatography paper can be successfully acetylated at room temperature, using appreciably larger concentrations of sulphuric acid as catalyst, so that minor variations in the purity of the acetylating reagents no longer produced marked variations in the properties of the papers produced.

The method is more fully described in the experimental section and gives papers of a very even degree of acetylation, and of excellent mechanical strength, which retain most of the fibre structure of the original paper. The $R_F$ values obtained with different batches of acetylated paper can be reproduced within close limits by exercising reasonable care in the acetylation process. The papers are sufficiently uniform to be developed by either the ascending or descending technique and show no variation in the rate of solvent travel across the width of the paper. The uniformity of the acetylation is of particular importance in the separation of compounds of similar, relatively low $R_F$ values, where the solvent front is allowed to over-run the end of the paper.

It is possible to vary the resolving power of the acetylated paper within wide limits by varying the catalyst, concentration and the time of acetylation. The papers produced vary between 21% and 31% acetyl content, but the separations obtained depend not only on the acetyl content of the paper, but also on the degree to which the fibre structure of the paper is retained. The greatest separations are obtained with those papers in which the fibre structure is well retained. Such papers, however, have a lower capacity than those in which some fibre structure has been destroyed with consequent swelling of the paper.

A summary of the $R_F$ values obtained for several polycyclic aromatic hydrocarbons on various types of acetylated paper is given in Tables I and II. The most satisfactory paper for the separation of two particular compounds depends both on the $R_F$ values of the compounds concerned and on the technique of identification used. Where the compounds are to be identified by comparison of $R_F$ values alone, and the distance of solvent travel is fixed, it is obvious that the papers of type I and II will give the best separation; but where the compounds are to be identified by extracting the spots and determining the ultra-violet absorption spectra of the extracts, the ratio of the $R_F$ values becomes of greater importance than their absolute values. The solvent front can be allowed to over-run the end of the paper and the greatest separations are then governed by the ratio of the two $R_F$ values and by the absolute mag-

TABLE I

$R_F$ VALUES FOR VARIOUS TYPES OF PAPER

| *Compound* | *$R_F$ values for paper type* | | | |
|---|---|---|---|---|
| | *I* | *II* | *III* | *IV* |
| 3:4-Benzopyrene | 0.240 | 0.186 | 0.115 | 0.055 |
| Chrysene | 0.460 | 0.410 | 0.272 | 0.164 |
| Perylene | 0.630 | 0.524 | 0.410 | 0.250 |
| Pyrene | 0.765 | 0.630 | 0.552 | 0.365 |
| Phenanthrene | 0.801 | 0.680 | 0.610 | 0.400 |

Solvent: methanol–ether–water, (4:4:1, v/v). 35 cm solvent ascent.

Catalyst concentration: based on 700 ml acetic anhydride and 1500 ml of thiophen-free benzene.

| | |
|---|---|
| Paper I | 1.6 g 92% sulphuric acid (21.2% acetyl content). |
| Paper II | 2.0 g 92% sulphuric acid (23.7% acetyl content). |
| Paper III | 2.5 g 92% sulphuric acid (25.1% acetyl content). |
| Paper IV | 3.4 g 92% sulphuric acid (28.0% acetyl content). |

TABLE II

COMPARISON OF $R_F$ VALUES FOR PAPER IV WITH DIFFERENT SOLVENT SYSTEMS

| *Compound* | *$R_F$ values* | |
|---|---|---|
| | *A* | *B* |
| 3:4-Benzopyrene | 0.055 | 0.183 |
| Chrysene | 0.164 | 0.400 |
| 1:2-Benzanthracene | 0.260 | 0.500 |
| Perylene | 0.250 | 0.530 |
| 3:4-Benzophenanthrene | 0.352 | 0.570 |
| Pyrene | 0.400 | 0.640 |

Solvent A: methanol–ether–water (4:4:1, v/v).
Solvent B: ethanol–toluene–water (17:4:1, v/v).

nitude of the higher $R_F$. Papers of type IV will, then, often give better separations of particular compounds, although the absolute $R_F$ values are considerably less than those obtained with papers of types I and II. For identification by this method, the papers are invariably run by the descending technique, and uniform acetylation of the papers is, therefore, of particular importance. When compounds of very low $R_F$ value are being separated by this method, it is possible to decrease the amount of streaking obtained by attaching a strip of ordinary chromatography paper to the end of the acetylated paper, so that the solvent supply to the acetylated paper is metered by passing through the untreated paper before reaching the actual chromatogram. The rate of solvent travel is reduced, and the spots are more discrete.

The position of the compounds on the developed chromatograms may usually be determined by viewing the papers under ultra-violet light, and the position of non-fluorescent compounds determined by spraying with a solution of tetrachlorophthalic anhydride[1]. Both high pressure and low pressure mercury lamps are normally used for exciting the fluorescence of the compounds as chrysene, phenanthrene, the phenylphenanthrenes and some other simple polycyclic compounds are not fluorescent under the usual high pressure mercury lamp with Woods glass filter, while coronene is difficult to locate under a low pressure lamp filtered to transmit mainly the 2537 Å mercury line. However, certain types of compounds, such as dibenzyl, stilbene, the isomeric phenylphenanthrenes, phenanthrene, and simple derivatives of naphthalene, are difficult to locate by these techniques when they are present in relatively low concentration. Similarly, a weakly fluorescent compound present in relatively large amounts may often be masked by the presence of a more strongly fluorescent compound of similar $R_F$ value. Such compounds are often more easily located by a modification of the photo-print technique described by MARKHAM AND SMITH[2]. The light source used is a 15-W low pressure mercury lamp without a filter, and the chromatogram held in firm contact with a strip of photographic paper sensitive to ultra-violet light by a quartz plate, is exposed and developed in the usual way. Similar techniques have been extensively used for several types of compounds chromatographed on cellulose, and partially acetylated paper appears to be almost as transparent to ultra-violet light at 2537 Å as ordinary chromatography paper, so that special techniques of rendering the paper transparent are unnecessary. The light emitted from the low-pressure lamp consists essentially of the 2537 Å mercury line, and it has been found that no advantage is gained by filtering the small percentage of light of longer wavelength. Fluorescent compounds may also be located by this method, the photographic paper being insensitive to the emitted light; but the location of these compounds by their fluorescence on the chromatogram is appreciably more sensitive than the photographic technique.

The well-known technique of locating compounds on a developed chromatogram by the use of a spectrophotometer[3] has also been applied successfully to partially acetylated paper. Most polycyclic aromatic hydrocarbons have intense absorption bands in the 2500–2800 Å region and are readily located by this method, although strongly fluorescent compounds sometimes appear as areas having a negative absorption relative to the background absorption. The technique has been fully described for the location of compounds on ordinary chromatography paper, but it should be pointed out that the success of the method depends on obtaining a constant reading for the absorption of the chromatogram itself, thus requiring an evenly acetylated paper. The papers produced by the method described below are particularly suitable for this type of location and show little variation in background absorption, whereas papers acetylated at 60 to 70° tend to give a variable reading which makes the location of small concentrations of compounds difficult. However, the photographic technique already described is equally as sensitive as the spectrophotometric location, which appears to offer no marked advantage.

While the techniques described have proved satisfactory for the identification of many compounds, it is often desirable to complete the identification of a compound from its melting point. Obviously it is possible to obtain milligram quantities of a compound by running a sufficiently large number of chromatograms, but it has been found that it is possible to isolate certain compounds from the extracts obtained from a limited number of chromatograms by using the technique of microsublimation. As the amounts of material concerned are small, the "sublimations" are carried out at atmospheric pressure using a very short path length, and the compounds are sublimed directly onto a microscope cover slip; the melting point is then determined in the usual way on a hot-stage microscope. Using an ordinary microscope slide with a depression into which the compound is introduced, and subliming onto a microscope cover slip covering the depression, it is possible to sublime hydrocarbons boiling as high as 500° at temperatures of 150–170° at atmospheric pressure. Where the compound has been extracted from a large area of paper it is necessary to rechromatograph the extract before sublimation in order to reduce the amount of resinous material and fibre in the extract. The technique is not invariably successful, particularly if the compound is relatively low-melting, but in many cases good melting points can be obtained. The technique may also be used to purify an extract before determining the ultra-violet absorption spectrum, thus reducing the amount of background absorption.

## CHROMATOGRAPHY OF SUBSTITUTED POLYCYCLIC HYDROCARBONS ON ACETYLATED PAPER

The separation of aromatic hydrocarbons containing a single alkyl grouping is a problem of particular difficulty and is of importance in the study of the free radical reactions of hydrocarbons. Thus, although SZWARC[4] has studied the relative rates of substitution of many aromatic hydrocarbons by methyl radicals, it has not been possible to establish the position of the methyl grouping in the substituted compounds. The $R_F$ values obtained for methyl substituted 1:2-benzanthracenes and benzo-(c)-phenanthrenes are summarised in Table III, where the $R_F$ values have been referred to 3:4-benzopyrene as standard, since this method of comparison appears to give more reproducible results than do the absolute $R_F$ values. The separations recorded are for a given distance of solvent travel, and may be greatly increased by allowing the solvent front to over-run the end of the paper. It is apparent that the position of the methyl grouping in the two molecules studied can often be assigned unequivocally, and can be limited to two positions in the most unfavourable cases. It is interesting to observe that although the sterically hindered isomers of 1:2-benzanthracene, the 1'- and 9-methyl, have the lowest $R_F$ values of this group, the same is not true of the benzo-(c)-phenanthrenes, where the parent molecule already has a non-planar structure.

TABLE III

$R_F$ VALUES FOR ACETYLATED PAPER TYPE IV

| *Compound* | *$R_F$ (relative to 3:4-benzopyrene)* | |
|---|---|---|
| | *A* | *B* |
| Benzo-(c)-phenanthrene | 2.96 | 5.86 |
| 1-Methyl-benzo-(c)-phenanthrene | 2.64 | 5.20 |
| 2-Methyl-benzo-(c)-phenanthrene | 3.38 | 6.95 |
| 3-Methyl-benzo-(c)-phenanthrene | 2.90 | 5.76 |
| 4-Methyl-benzo-(c)-phenanthrene | 2.40 | 3.47 |
| 5-Methyl-benzo-(c)-phenanthrene | 3.25 | 5.84 |
| 6-Methyl-benzo-(c)-phenanthrene | 3.66 | 7.44 |
| 1:2-Benzanthracene | 2.65 | 4.44 |
| 3-Methyl-1:2-benzanthracene | 3.30 | 5.90 |
| 4-Methyl-1:2-benzanthracene | 2.82 | 5.00 |
| 5-Methyl-1:2-benzanthracene | 2.74 | 3.80 |
| 6-Methyl-1:2-benzanthracene | 3.00 | 4.52 |
| 7-Methyl-1:2-benzanthracene | 3.45 | 5.95 |
| 9-Methyl-1:2-benzanthracene | 1.93 | 2.94 |
| 10-Methyl-1:2-benzanthracene | 2.80 | 3.64 |
| 1'-Methyl-1:2-benzanthracene | 2.04 | 2.50 |
| 2'-Methyl-1:2-benzanthracene | 2.98 | 5.25 |
| 3'-Methyl-1:2-benzanthracene | 3.20 | 5.70 |
| 4'-Methyl-1:2-benzanthracene | 2.50 | 3.34 |

Solvent A: ethanol–toluene–water (17:4:1, v/v).
Solvent B: methanol–ether–water (4:4:1, v/v).

Similar separations can be expected on columns of partially acetylated cellulose, but unfortunately, insufficient material was available to test this hypothesis fully.

From the results obtained it is apparent that the separation of methyl substituted polycyclic aromatic hydrocarbons by chromatography on partially acetylated cellulose may be of considerable assistance in the study of free radical reactions between aromatic hydrocarbons and alkyl radicals.

## ACETYLATION TECHNIQUE

Sixteen strips of Whatman's chromatography paper (No. 1), 24 in. by 6 in. were rolled together and immersed in an acetylating mixture comprising acetic anhydride (redistilled, 700 ml), thiophen-free benzene (1500 ml) and sulphuric acid (92%, 2.0 to 3.0 g) and allowed to stand for 24 h at 18° with frequent stirring and separation of the papers. After this time the acetylating mixture was poured off, the papers allowed to drain, and then allowed to stand for 24 h in ethanol, with occasional stirring. The papers were washed with a further quantity of ethanol and then in running water for 6 h and finally allowed to stand in distilled water for 2 h. After this time the papers were removed and air-dried.

The acetylating mixture can be used for another two batches of paper, with the addition of 0.5 g of fresh sulphuric acid for each successive batch, before the papers become unsuitable through destruction of the fibre structure; the properties of the successive batches are different but all give good resolutions. Where reproducible results are desired, fresh acetylating mixture should be used for each acetylation.

## CHROMATOGRAPHY OF POLYCYCLIC AROMATIC HYDROCARBONS ON A COLUMN OF PARTIALLY ACETYLATED CELLULOSE

For many years the method of choice for the purification and separation of polycyclic aromatic hydrocarbons has been by liquid phase chromatography on alumina, both because of the high resolution obtained and the large capacity of the columns.

However, in the analysis of complex mixtures of hydrocarbons obtained in pyrolytic reactions, it has been found that it is extremely difficult to effect good resolutions of the higher polycyclic fractions, which often contain six or seven compounds, by this method. Chromatography of the fractions obtained from an alumina column by the conventional methods, on a column of partially acetylated cellulose has been found to give excellent resolutions of the compounds present in such fractions, and the number of compounds present in each fraction limited to two at the most and usually to a single major compound. The separations obtained are not usually as complete as those obtained by paper chromatography on partially acetylated paper, mainly because of the deficiencies of a dynamic method of resolution as opposed to a static method, but in conjunction with paper chromatography, the method provides a powerful analytical tool for the analysis of these fractions. While the separations obtained for compounds of relatively high $R_F$ are not as efficient as those obtained by paper chromatography, the resolution obtained for compounds of low $R_F$ are often better because of the increased distance available for resolution. Thus, it is possible to obtain complete resolution of 3:4-benzopyrene, which has a particularly low $R_F$, from the compounds accompanying it in some pyrolysis fractions, such as perylene, 1:2-benzopyrene, chrysene and 3:4-benzofluoranthene.

The technique of acetylation and the method of packing the column are described in the experimental section. Good resolutions of polycyclic aromatic hydrocarbons have been obtained on columns of 24% and 32% acetyl content, the more highly acetylated cellulose giving more discrete bands and also retaining the compounds for a shorter time. The solvent systems used for the development of the columns, and also for the introduction of the compounds onto the column, were similar to those used for paper chromatography on partially acetylated paper, namely ethanol–benzene–water (17:4:1, v/v), ethanol–toluene–water (17:4:1, v/v) and methanol–toluene–water (10:1:1, v/v). The solvent systems containing ethanol have a greater eluting power than that containing methanol, but the latter is often useful in effecting increased resolution of the compounds of relatively high $R_F$ value, particularly on the column of lower acetyl content. Some variation in $R_F$ values was observed between the three solvent systems, but in the majority of cases the order of elution was the same.

The presence of water in the eluting solvent increases the rate at which the compounds move through the column and also serves to keep the limits of the individual bands sharply defined. While good resolutions have been obtained using mixtures of ethanol and toluene, and ethanol and benzene alone, the proportion of hydrocarbon in the eluting solvent being gradually increased during development, the absence of water causes extensive tailing of the bands, so reducing the effectiveness of the separations. However, compounds differing in $R_F$ by 0.15 to 0.2 or more have been resolved with these solvent systems.

With the three solvent systems described above, it is possible to resolve compounds having an $R_F$ difference of 0.08 with an absolute $R_F$ value greater than 0.4, and compounds with an $R_F$ difference of 0.05 with an absolute $R_F$ value less than 0.4 on a column of partially acetylated cellulose (32% acetyl content) 70 cm long and 2.5 cm diameter. The capacity of the column is low, and it has been found that approximately 200 g of partially acetylated cellulose is required to give good resolution of 400 mg of a mixture of polycyclic aromatic hydrocarbons having a range of $R_F$ values. The efficiencies of the separations obtained depend not only on the ratio of the $R_F$ values of the two compounds concerned, but also on the extent to which the bands tail during development. Fluoranthene and 9,10-diphenylanthracene are particularly bad in this respect, and are often difficult to separate from the compounds of similar $R_F$.

The order of elution from a column of partially acetylated cellulose is given in Table IV, together with the $R_F$ values obtained using the same solvent system on partially acetylated papers of slightly lower acetyl content (28.0%), and it will be seen that the order of elution follows the order of $R_F$ values, as would be expected. It is obvious that many compounds have similar $R_F$ values and cannot be satisfactorily resolved by this means, but in most cases these compounds can be separated by first effecting a preliminary separation on an alumina column by the usual methods, or by fractional distillation. The technique is of particular value in the isolation of small amounts of the higher polycyclic aromatic hydrocarbons containing five or more rings, which are eluted almost simultaneously from an alumina column. The order of elution from a column of activated alumina with a solvent of continuously increasing strength, from hexane to benzene, is given in Table V. In practice it has been found that the complex mixtures of hydrocarbons obtained in pyrolytic reactions of hydrocarbons can be satisfactorily fractionated by distillation of the fractions boiling below 280° and by chromatography of the residue on activated alumina followed by chromatography of the fractions on partially acetylated cellulose. Where the fractions obtained from the column of partially acetylated cellulose contain two compounds, the fractionation may usually be completed by using the techniques of paper chromatography on partially acetylated paper already described, allowing the solvent front to over-run the end of the paper to obtain maximum resolution. The division of a fraction into groups of compounds of approximately the same $R_F$ value is also of advantage in the analysis of the fraction by paper chromatography since the type of

TABLE IV

ORDER THROUGH A COLUMN OF PARTIALLY ACETYLATED CELLULOSE

Solvent: ethanol–toluene–water (17:4:1, v/v).

| *Compound* | $R_F$ |
|---|---|
| 9,10-Diphenylanthracene | 0.675 |
| Acenaphthylene | 0.576 |
| 2:3-Benzofluorene | 0.539 |
| 1,1′-Dinaphthyl | 0.539 |
| Phenanthrene | 0.501 |
| Anthracene | 0.494 |
| 3:4-Benzophenanthrene | 0.483 |
| Pyrene | 0.471 |
| Triphenylene | 0.446 |
| Fluoranthene | 0.441 |
| Coronene | 0.410 |
| Fluorene | 0.401 |
| 1:2-Benzanthracene | 0.396 |
| 2,2′-Dinaphthyl | 0.359 |
| Perylene | 0.351 |
| 1:2:5:6-Dibenzanthracene | 0.246 |
| 3:4-Benzofluoranthene | 0.209 |
| 3:4:9:10-Dibenzopyrene | 0.209 |
| Chrysene | 0.206 |
| Anthanthrene | 0.163 |
| 3:4-Benzopyrene | 0.093 |
| 2:3:10:11-Dibenzoperylene | 0.042 |
| Violanthrene | 0.023 |

TABLE V

ORDER THROUGH ACTIVATED ALUMINA COLUMN

Solvent: *n*-hexane with gradually increasing benzene concentration.

Acenaphthene
Acenaphthylene
Fluorene
Phenanthrene
Anthracene
Pyrene
Fluoranthene
1:2-Benzanthracene
Chrysene
1:2:5:6-Dibenzanthracene
Perylene
3:4-Benzopyrene
3:4:9:10-Dibenzopyrene
Coronene
2:3:10:11-Dibenzoperylene
Violanthrene

acetylated paper can then be chosen so that maximum resolution is obtained. Thus it has been found that a paper of relatively high acetyl content (27–29%) is most suitable for the separation of compounds having a high $R_F$ and for compounds of relatively low $R_F$, the best results are obtained with papers of lower acetyl content (20–24%).

Similar separations have been obtained from columns prepared by pulping partially acetylated papers, but the difficulties encountered in packing the fibrous mass obtained into an efficient column usually causes extensive over-lapping of the fractions.

Chromatography on partially acetylated cellulose columns is not proposed as an alternative method to the more usual technique of chromatography on activated alumina, but as an adjunct to this technique by which many difficult resolutions can be effected more easily. The $R_F$ values recorded for methyl substituted 1:2-benzanthracenes and benzo-(c)-phenanthrenes (Table III) suggest that the technique may be of considerable assistance in the analysis of mixtures of these compounds. Complete resolutions of 4-, 7- and 5-methyl-benzo-(c)-phenanthrene and of 6-, 5-, 4- and 3-methyl-1:2-benzanthracene have been obtained on a column 70 cm long and 2.5 cm in diameter.

## EXPERIMENTAL

### *Acetylation of cellulose powder*

Whatman's cellulose chromatography powder (200 g) was acetylated in a mixture of thiophen-free benzene (1700 ml) and acetic anhydride (800 ml) containing 4.5 g of 92% sulphuric acid. The mixture was stirred for 24 h at 18°, filtered and the acetylated pulp stirred with 2,500 ml of ethanol for 4 h. The pulp was again filtered and stirred with a further 2,500 ml of ethanol for 5 h, filtered and dried at 70°.

The acetyl content of the powder was found to be 32%. An acetylation carried out under the same conditions using 3.0 g of 92% sulphuric acid as catalyst gave a pulp of 24% acetyl content.

### *Preparation of columns*

The partially acetylated cellulose was slurried with the developing solvent in a Waring blender and the columns packed by pouring the slurry into a suitable column and allowing it to settle with intermittent vibration. The density of the partially acetylated pulp is considerably greater than that of cellulose powder, and a coherent column of good density can be obtained without compressing the powder. The columns can be used repeatedly without any obvious decrease in efficiency.

The use of a short column of smaller diameter to straighten the fronts of the eluates is an advantage in increasing the sharpness of the fractionation.

In establishing the order of elution from these columns the purity of the fractions obtained was determined by chromatography on partially acetylated paper.

## ACKNOWLEDGEMENTS

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## SUMMARY

The techniques previously described[1] for the separation of polycyclic aromatic hydrocarbons by chromatography on acetylated paper have been supplemented by the introduction of alternative methods of locating compounds on the developed chromatograms, by the isolation of the compounds in crystalline form from extracts from paper chromatograms, and by the use of an improved technique of acetylation which has been found to produce papers of increased resolving power and better mechanical properties.

The separations obtained on partially acetylated paper have been reproduced on the larger scale on columns of partially acetylated cellulose powder, using the same solvent systems as those used in the paper chromatography of these compounds.

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*Note added in proof*

The method of acetylation of cellulose powder described in this paper has proved unsatisfactory with some batches of Whatman's standard chromatography powder, which do not acetylate appreciably under these conditions. However, the following catalysts, based on the acetylating mixture already described, gave a satisfactory product.

I. 92% sulphuric acid (4–5 g) with 72% perchloric acid (1 g).
II. 92% sulphuric acid (4 g) with 72% perchloric acid (4 g).
III. 72% perchloric acid (10 g), acetylated for 10 hours only.

These methods have been developed in collaboration with Dr. M. J. THOMPSON of this Department.

# ANALYSE CHROMATOGRAPHIQUE DE L'ACTION LÉCITHINASIQUE DU VENIN DE COBRA ET D'ABEILLE

M. A. CIASCA RENDINA ET F. BOVET-NITTI

*Laboratoire de Chimie Thérapeutique, Institut Supérieur de Santé, Rome (Italie)*

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Dans des travaux antérieurs l'une de nous[1] a montré la possibilité d'appliquer la méthode manométrique de Warburg à la mesure de l'action enzymatique du venin de cobra vis à vis de la lécithine et d'autres esters gras de la choline. Le fait de pouvoir suivre en quelque sorte la cinétique du phénomène nous apparaissait comme un progrès par rapport aux méthodes utilisées jusqu'à ce jour et qui ne permettaient de mesurer que l'entité de la réaction accomplie. C'est dans le même esprit que nous nous sommes demandé s'il n'allait pas être possible de suivre cette réaction, outre que par la technique manométrique, par une analyse chromatographique des contenus des flacons de Warburg, ainsi que l'ont fait WHITTAKER ET WIJESUNDERA[2] dans le cas de l'hydrolyse de la succinylcholine par la cholinestérase sérique.

Si de nombreux auteurs se sont en effet servis de l'éléctrophorèse sur papier pour séparer les diverses fractions actives des venins de cobra (NEUMANN[3], HABERMANN ET NEUMANN[4], GRASSMANN ET HANNIG[5]) et d'abeille et si BUSSARD[6] a tenté par une methode analogue d'élucider la constitution du venin de *Naja-Naja*, l'on ne trouve pas à notre connaissance dans la littérature de travaux portant sur l'analyse chromatographique ou éléctrophorétique de l'action enzymatique elle même exercée par les venins de serpents et d'insectes, si l'on excepte un travail très récent[7] relatant l'action du venin de Crotale sur la lécithine du coeur de boeuf et l'examen par la chromatographie des résidus de plusieurs jours. Si le venin de Crotale[8] a fait l'objet de nombreux travaux quant à sa nature, le détail de son action enzymatique reste encore à élucider en ce qui concerne les produits de dégradation. L'on sait toutefois qu'il est denué d'action cholinestérasique[9] et que, contrairement au venin de cobra, il exerce une action sur la coagulation du sang[10].

## PARTIE EXPÉRIMENTALE

### *Venins employés*

Pour tous nos essais avec le venin de cobra nous nous sommes exclusivement servis d'un échantillon de venin de *Naja-Naja* provenant du Haffkine Institute de Bombay*. Dans le cas de venin d'abeille nous avons nous mêmes procédé à l'extraction des glandes d'un certain nombre d'insectes qui ont été ensuite desséchées à l'étuve à 37° et finement pulverisées.

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* Nous remercions vivement le Colonel S. S. BATHNAGAR qui nous a fourni le venin de cobra.

### *Substrat*

Comme source de lécithine nous avons eu recours à une dilution de jaune d'oeuf à 10%.

### *Technique manométrique*

L'hydrolyse de la lécithine a été suivie par la méthode de Warburg dans des conditions très voisines de celles décrites précédemment[1]. Ringer 20, température à 30°. Toutes les solutions contenaient 0.2% de $NaHCO_3$, le volume total étant dans chaque flacon de 2 c.c. en tout. Le substrat (1.5 c.c.) était placé au milieu et l'enzyme (0.5 c.c.) dans le bulbe latéral. Les dilutions s'entendent pour la totalité du liquide mis en oeuvre. Les lectures ont été faites cinq minutes après mise en contact du substrat et de l'enzyme et ensuite aux intervalles indiqués pour le détail de chaque expérience. Pour arrêter la réaction enzymatique les contenus des flacons étaient immédiatement placés dans des éprouvettes maintenues dans la neige carbonique et portées ensuite à — 20°.

### *Chromatographie ascendante sur papier*

*Lécithine et lysocithine.* Après de nombreux essais nous avons en définitive adopté la technique décrite par KIRCHNER ET KELLER[11] et rapportée par LEA, RHODES ET STOLL[12] et dont la modification essentielle sur les méthodes employées auparavant consiste dans le fait d'imprégner le papier (Whatman No. 3MM) d'acide silicique sec. Les méthodes essayées précédemment[13-15] comme on le voit d'après le Tableau I, ne permettaient pas en effet une séparation assez nette des deux produits.

TABLEAU I

VALEURS DES $R_F$ OBTENUS PAR LA CHROMATOGRAPHIE DES PRÉLÈVEMENTS DES RÉSIDUS DE FLACONS DE WARBURG AU COURS DE L'HYDROLYSE DE LA LÉCITHINE DU JAUNE D'OEUF PAR LE VENIN DE COBRA, SUIVANT LES DIVERSES TECHNIQUES EMPLOYÉES

| *Papier employé* | *Solvant* | *Lécithine* $R_F$ | *Lysocithine* $R_F$ |
|---|---|---|---|
| Whatman No. 1 non traité[13] | Alcool amylique–acétone (7:3) | 0.70 | 0.60 |
| Whatman No. 1 non traité[14] | Chloroforme–éthanol–eau (80:20:2.5) | 0.90 | 0.90 |
| Whatman No. 1 non traité[15] | Éthanol–eau (8:1) | 0.90 | 0.90 |
| Whatman No. 1 non traité[15] | *n*-Butanol saturé d'eau | 0.85 | 0.85 |
| Whatman No. 3MM impregné d'acide silicique[12] | Méthanol–chloroforme (20:80) | 0.78 | 0.20 |

Le papier Whatman No. 3MM, format 12.5 × 12.5 était imprégné d'acide silicique sec. Le solvant employé est du méthanol–chloroforme 20 : 80 sur lequel les papiers devant servir aux chromatogrammes sont laissés pendant une demie heure avant le développement[12]. La quantité du liquide prélevé et déposée sur le papier était de 0.15 c.c. Après développement les chromatographies ont été révélées par la méthode de LEVINE ET CHARGAFF[16] décrite pour les phospholipides contenant de la choline.

*Acides gras.* Afin de suivre complètement la réaction enzymatique aboutissant, outre qu'à la formation de lysocithine, également à la libération d'un acide gras, nous avons extrait les contenus des flacons de Warburg trois fois par le chloroforme et avons ensuite effectué les chromatographies sur du papier à la paraffine suivant la technique de SPITERI[17]. Après développement l'on met en évidence les acides gras en les précipitant sous forme de sels de plomb par la coloration brune qu'ils donnent en présence de $H_2S$[18].

### 1. *Hydrolyse de la lécithine du jaune d'oeuf par le venin de cobra*

Dans une prémière série d'expériences, utilisant les conditions expérimentales précédemment rapportées par l'une de nous, nous avons confirmé les observations montrant la rapidité de l'hydrolyse de la lécithine par le venin de cobra.

En mettant en présence dans un manomètre de Warburg une émulsion de jaune d'oeuf à 10% avec des dilutions de venin allant d'une concentration de $10^{-3}$ à $10^{-8}$ dans un milleu à pH 7.4 riche en bicarbonate, l'on observe dans les premières 20 minutes de l'expérience un dégagement de $CO_2$ correspondant à l'hydrolyse de la fonction ester et à la formation de l'acide gras.

À titre d'exemple, les valeurs trouvées pour l'expérience représentée graphiquement dans la Fig. 1 sont données dans le Tableau II.

TABLEAU II

| *Concentration du venin de cobra* | *$mm^3$ de $CO_2$ dégagé en* | | | | | | |
|---|---|---|---|---|---|---|---|
| | *5 min* | *10 min* | *20 min* | *30 min* | *40 min* | *50 min* | *60 min* |
| $10^{-3}$ | 128 | 215 | 234 | 237 | 237 | 237 | 237 |
| $10^{-4}$ | 105 | 178 | 220 | 236 | 240 | 240 | 243 |
| $10^{-5}$ | 65 | 128 | 155 | 177 | 192 | 192 | 197 |
| $10^{-6}$ | 45 | 60 | 87 | 94 | 98 | 107 | 116 |
| $10^{-7}$ | 0 | 12 | 14 | 24 | 28 | 33 | 38 |
| $10^{-8}$ | 0 | 12 | 14 | 13 | 16 | 13 | 16 |

Poursuivant l'expérience nous avons procédé à l'analyse chromatographique des contenus des flacons de Warburg prélevant le mélange de venin et de lécithine après des périodes déterminées d'incubation et interrompant à chaque fois la réaction par refroidissement à —20°.

Les chromatographies réalisées avec la méthode de LEA, RHODES ET STOLL, mettent en évidence la disparition progressive de la lécithine et sa transformation en lysocithine. Le parallélisme entre les résultats fournis par la méthode manométrique et l'analyse chromatographique apparaît clairement par la comparaison des données numériques de l'expérience et des chromatogrammes rapportées dans la Fig. 2; l'on observe la disparition rapide de la lécithine et sa transformation en lysocithine pour des concentrations de venin de cobra allant jusqu'à $10^{-4}$. À partir d'une concentration de $10^{-5}$ cette transformation est progressive et n'est complète qu'au bout d'une heure. À $10^{-6}$ le venin, au cours des dix premières minutes, forme des traces assez visibles de lysocithine, à $10^{-7}$ la trace de lysocithine n'apparaît qu'au bout d'une heure de mise en contact et à $10^{-8}$ l'on ne voit plus de formation de lysocithine.

Dans d'autres expériences l'utilisation de la méthode de SPITERI[17] nous a permis de mettre en évidence au cours de l'effet du venin sur les lécithines du jaune d'oeuf une tâche indiquant la formation d'un acide gras et dont l'apparition est parallèle à

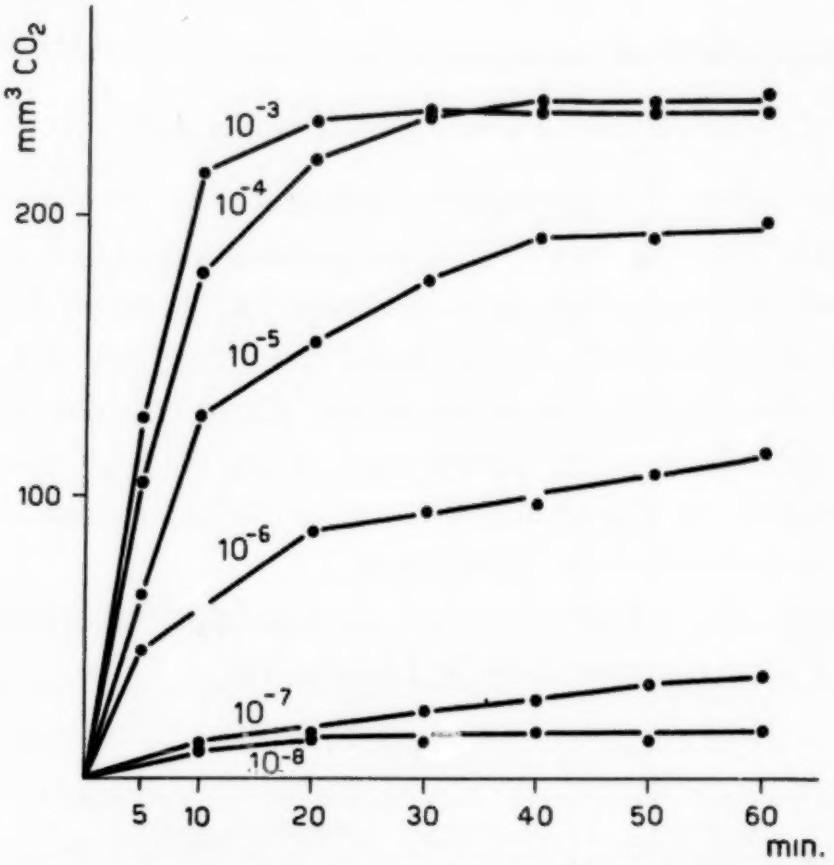

Fig. 1. Effet de différentes concentrations de venin de cobra sur une dilution de lécithine du jaune d'oeuf. Méthode manométrique de Warburg. Ringer 20, température 30°. En abscisse sont indiquées les intervalles de temps en minutes et en ordonnée les mm³ de $CO_2$ dégagé.

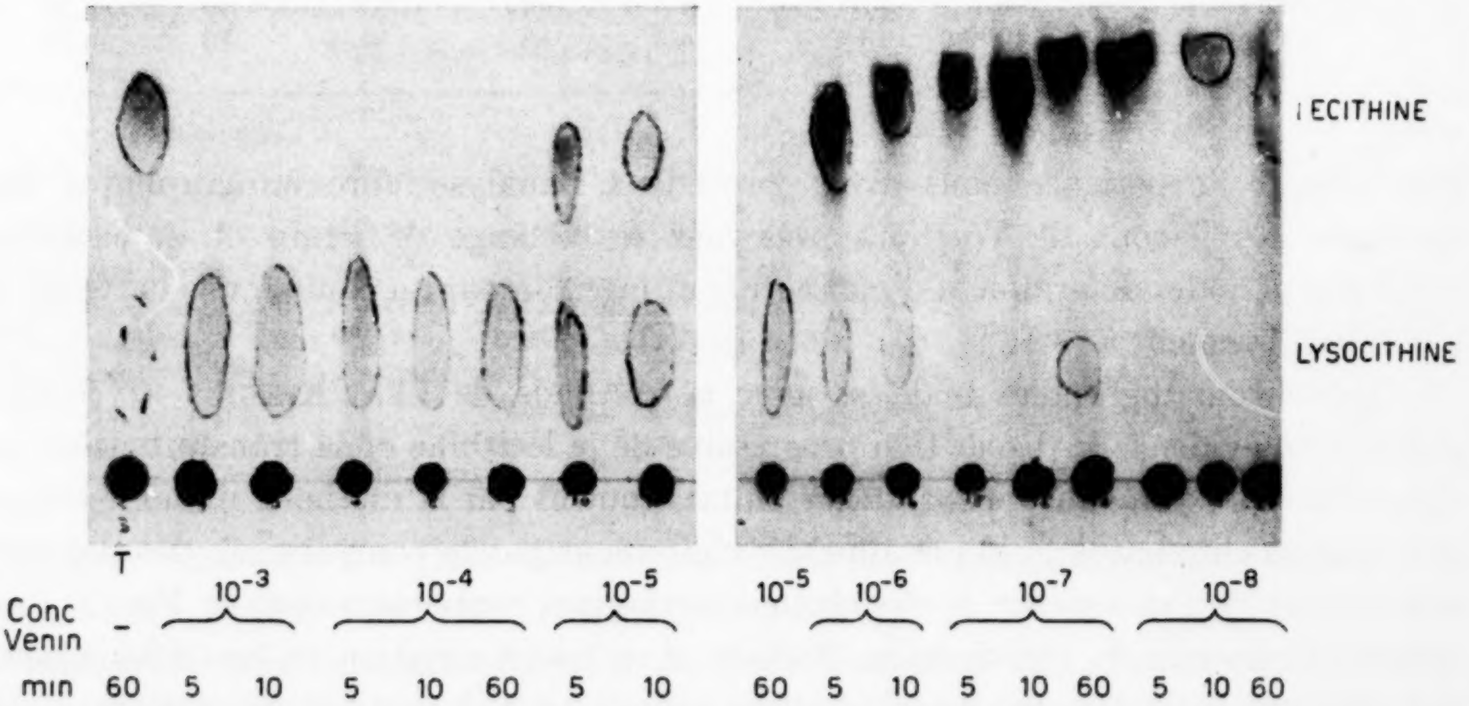

Fig. 2. Chromatographie ascendante de la lécithine du jaune d'oeuf et de la lysocithine formée au cours de son hydrolyse enzymatique par le venin de cobra. T = dilution de jaune d'oeuf à 10% après incubation à 30° et agitation pendant 60 min. Les chromatogrammes successifs correspondent aux dilutions de jaune d'oeuf à 10% après incubation à 30° en présence de concentrations décroissantes de venin de cobra ($10^{-3}$ à $10^{-8}$) et pendant des intervalles de temps déterminés (5 à 60 min).

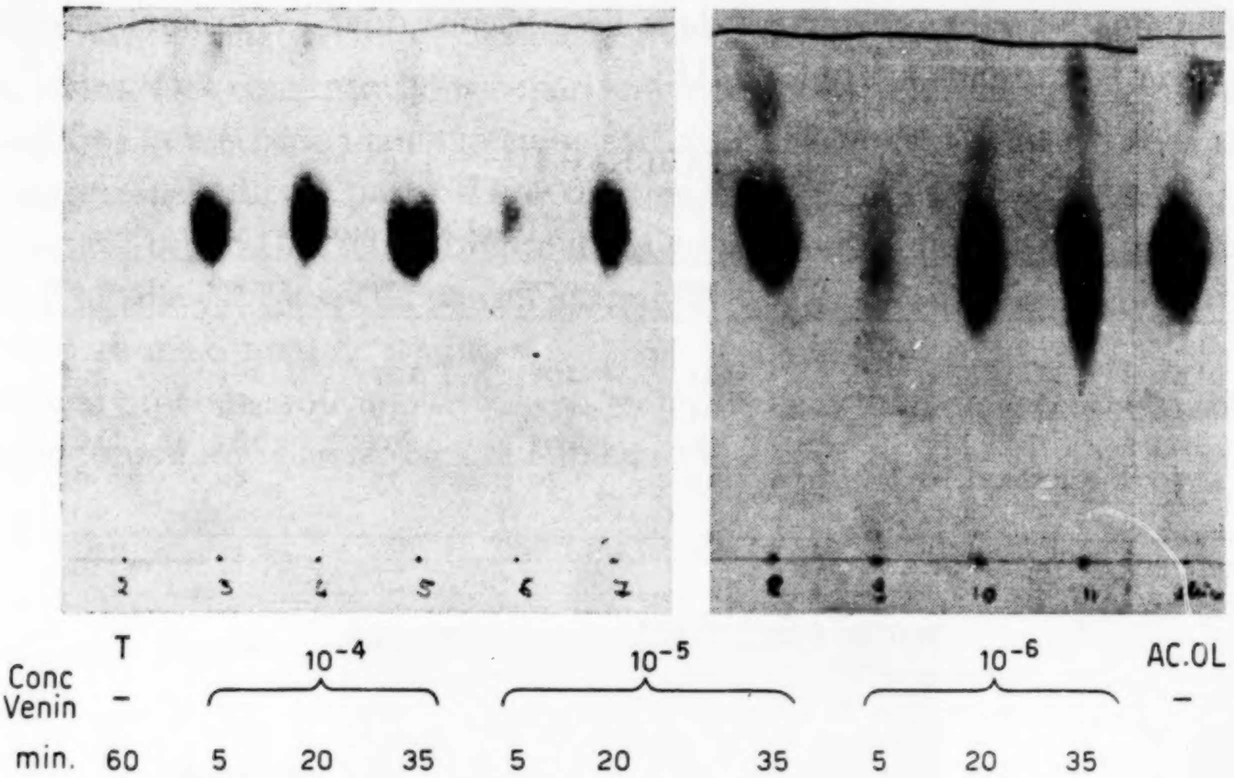

Fig. 3. Chromatographie ascendante des acides gras (acide oléique) formés au cours de l'hydrolyse enzymatique de la lécithine par le venin de cobra. T = dilution de jaune d'oeuf à 10% après incubation à 30° et agitation pendant 60 min. AC.OL. = chromatogramme de l'acide oléique. Les chromatogrammes successifs correspondent aux dilutions de jaune d'oeuf à 10% après incubation à 30° en présence de concentrations de venin en ordre décroissant ($10^{-4}$ à $10^{-6}$) et pendant des intervalles de temps déterminés (5 à 35 min).

celle de la lysocithine et correspond exactement aux phases de la disparition progressive de la lécithine; elle indique l'apparition de l'acide—probablement l'acide oléique—libéré au cours de l'hydrolyse de la lécithine par le venin (Fig. 3).

### 2. *Action des inhibiteurs de la lécithinase; acide 4-aminophénylarsinique* (*Atoxyl*)

Si l'estérase contenue dans le venin de cobra peut être inhibée par de nombreuses substances[1] la lécithinase A est par contre très résistante aux inhibiteurs habituels. À la suite des travaux de WILLSTAETTER ET MEMMEN[19], de RONA ET AMMON[20] et de GYOTOKU[21] sur l'action inhibitrice de certains arsénicaux sur la lipase du sérum, du foie et de l'estomac et bien qu'il n'ait pas été décrit d'action lipasique du venin de cobra, nous avions nous mêmes étudié[22] l'action de quelques uns de ces dérivés sur la lécithinase A du venin de cobra. L'un des inhibiteurs qui mérite particulièrement de retenir l'attention est représenté par l'Atoxyl (acide 4-aminophénylarsinique) qui, dans nos essais antérieurs, s'est montré un antagoniste de l'action hémolytique du venin et a montré, au cours des essais réalisés avec la technique manométrique, une faible action inhibitrice de la lécithinase à des concentrations où il ne modifiait pas les propriétés estérasiques du venin. Nous avons trouvé intéressant d'en reprendre l'étude en associant la méthode manométrique à la recherche des produits de dégradation par l'analyse chromatographique.

À la concentration de $10^{-2}$, l'Atoxyl exerce sur la lécithinase de cobra une action inhibitrice qui se traduit par un ralentissement de l'hydrolyse de la lécithine, et une diminution de la quantité totale de la lécithine hydrolysée.

Nous donnons les valeurs trouvées dans l'expérience dont le chromatogramme fait l'objet de la Fig. 4 dans le Tableau III.

TABLEAU III

| *Concentration du venin de cobra* | *mm³ de CO₂ dégagé en* | | | | |
|---|---|---|---|---|---|
| | *5 min* | *10 min* | *20 min* | *30 min* | *40 min* |
| $10^{-4}$ | 124 | 190 | 197 | 201 | 205 |
| $10^{-4}$ + Atoxyl $10^{-2}$ | 64 | 116 | 155 | 162 | 187 |
| $10^{-5}$ | 50 | 93 | 150 | 163 | 165 |
| $10^{-5}$ + Atoxyl $10^{-2}$ | 16 | 50 | 75 | 82 | 82 |

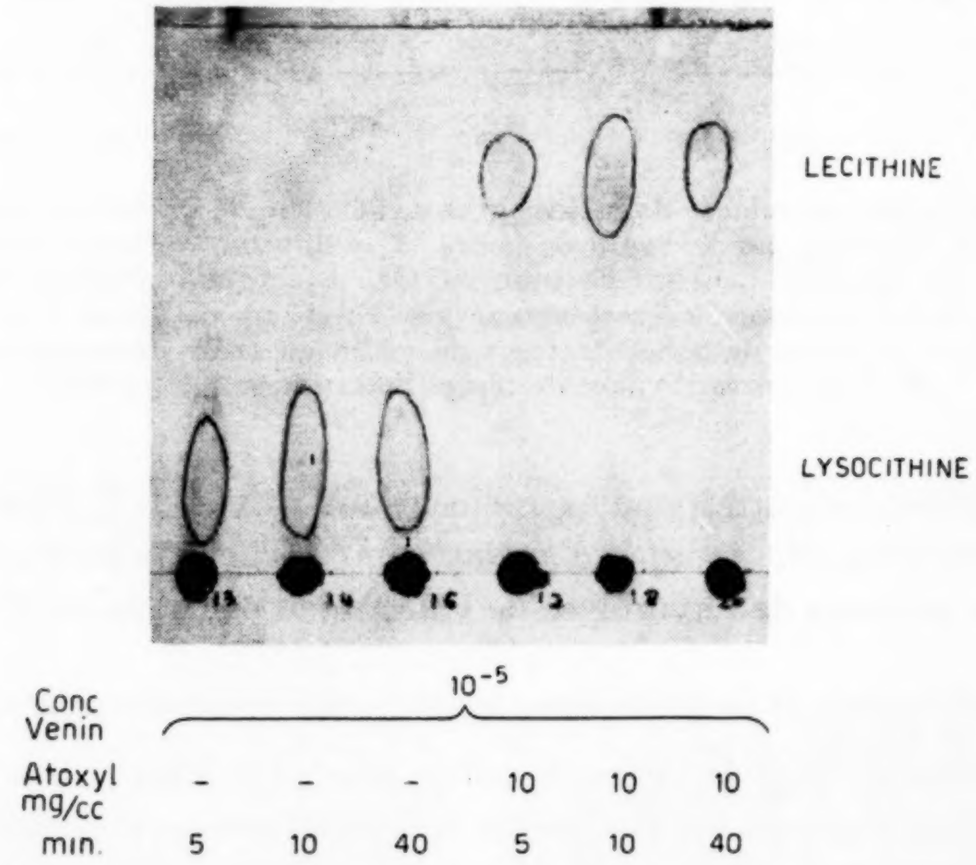

Fig. 4. Chromatographie ascendante de la lécithine du jaune d'oeuf et de la lysocithine formée au cours de son hydrolyse enzymatique par le venin de cobra sans et avec l'adjonction de 10 mg/c.c. d'Atoxyl intervenant comme inhibiteur de l'action lécithinasique. Les conditions expérimentales sont identiques à celles de la Fig. 2.

Dans des expériences réalisées parallèlement nous avons pu également observer que l'Atoxyl, en ralentissant ou en s'opposant à la formation de lysocithine, retarde également l'apparition de l'acide gras.

### 3. *Action lécithinasique du venin d'abeille* (*Apis mellifica*)

Si la littérature est assez riche en renseignements sur le venin d'abeille dans ses rapports avec l'immunologie, la thérapie de certains phénomènes douloureux et sur sa constitution[4], il faut se reporter aux anciens travaux de CONTARDI ET ERCOLI[23] pour trouver des précisions sur la lécithinase A qu'il contient. Nous avons eu recours à ce poison pour avoir une vérification supplémentaire de l'analyse chromatographique et avons en effet retrouvé des effets très comparables à ceux obtenus avec le venin de cobra. Des concentrations supérieurs à celles du venin de cobra sont nécéssaires pour que la méthode manométrique soit utilisable et ce venin s'avère pratiquement presque sans action à partir d'une dilution de $10^{-6}$. Dans l'interprétation de ces résultats il faut cependant tenir compte du fait que, tandis que le venin de cobra est pur, celui d'abeille est constitué par un extrait de la glande tout entière ne contenant qu'un quart au maximum de venin.

Dans une expérience où nous avons mis en jeu les deux venins contemporainement, nous avons trouvé les valeurs dans le Tableau IV.

TABLEAU IV

| *Concentrations des venins* | *mm³ de CO₂ dégagé en* | | | |
|---|---|---|---|---|
| | *5 min* | *20 min* | *40 min* | *60 min* |
| Cobra $10^{-5}$ | 65 | 152 | 182 | 187 |
| Abeille $10^{-5}$ | 38 | 54 | 64 | 83 |
| Cobra $10^{-6}$ | 43 | 87 | 98 | 116 |
| Abeille $10^{-6}$ | 7 | 17 | 25 | 25 |

L'étroite analogie entre les actions lécithinasiques exercées par les venins d'abeille et de cobra ressort également avec netteté de l'examen chromatographique des produits de la réaction.

La Fig. 5 montre une disparition progressive de la lécithine et l'apparition de la lysocithine avec des effets très superposables à ceux déjà observés dans le cas du

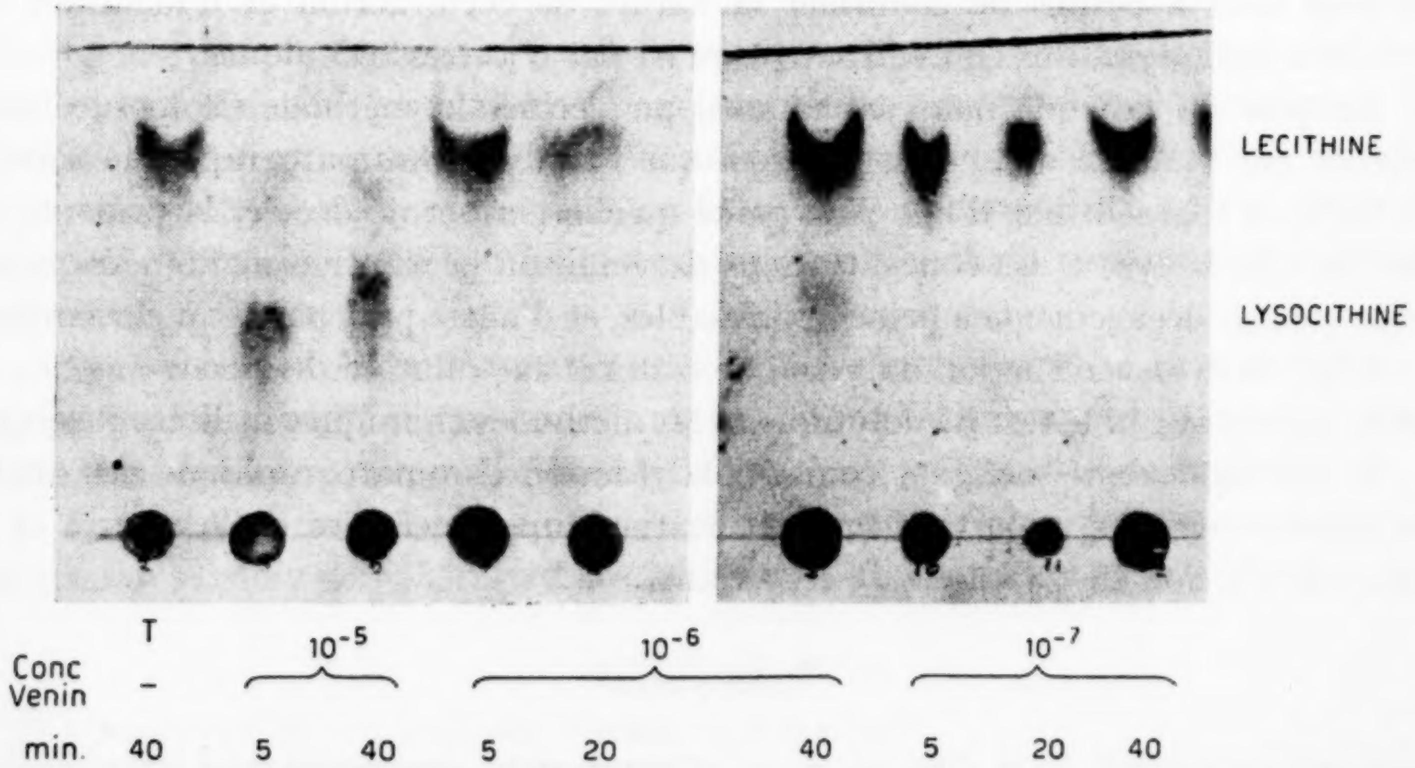

Fig. 5. Chromatographie ascendante de la lécithine du jaune d'oeuf et de la lysocithine formée au cours de son hydrolyse enzymatique par le venin d'abeille. Les conditions expérimentales sont analogues à celles de l'expérience rapportée à la Fig. 2.

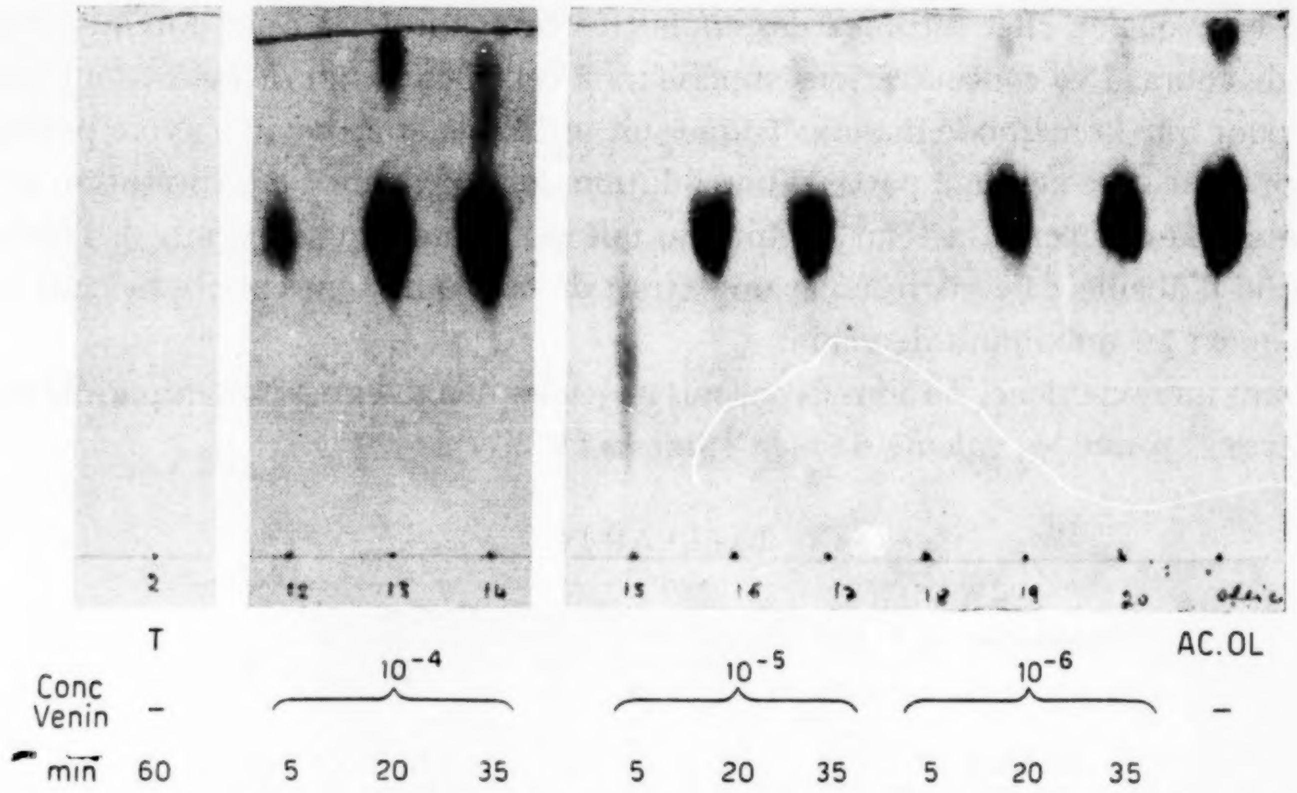

Fig. 6. Chromatographie ascendante des acides gras (acide oléique) formés au cours de l'hydrolyse enzymatique de la lécithine par le venin d'abeille. Conditions expérimentales identiques à celles de la Fig. 3.

venin de cobra. La présence de l'acide gras dégagé est également facilement identifiée par voie chromatographique (Fig. 6).

## CONCLUSIONS

L'analyse chromatographique de l'hydrolyse enzymatique de la lécithine du jaune d'oeuf par le venin de cobra et d'abeille suivie par la méthode manométrique de Warburg nous a permis de confirmer la nature de cette action et d'identifier les produits d'hydrolyse, mis en évidence jusqu'ici par d'autres techniques.

En plus du fait que nous avons ainsi pu vérifier la méthode de mesure déjà proposée par l'une de nous, il est apparû que l'analyse chromatographique s'avère très fidèle et très sensible, d'une part parce qu'elle permet de déceler la présence de produits d'hydrolyse à des concentrations de venin où la réaction manométrique ne donne que des dégagements à peine appréciables, et d'autre part parce qu'elle consent de mettre en évidence l'action du venin de cobra à une dilution de 0.0001 mg/c.c., ce que ne permettait ni le test hémolytique ni les méthodes chimiques utilisées jusqu'ici.

Il faut également souligner combien la réaction chromatographique des acides gras s'avère sensible, apportant une vérification supplémentaire de la nature et de l'allure de l'action enzymatique de ces venins.

## ADDENDUM

Alors que ce travail était déja en cours d'impression, nous avons eu connaissance d'une étude sur l'effet du venin de cobra sur la lécithine marquée en $^{32}P$ et isolée à partir du foie de rat (MARINETTI, ERBLAND ET STOTZ[24]). Ces mêmes auteurs ont également examiné l'action du venin sur une lécithine isolée à partir du jaune d'oeuf: une chromatographie sur papier impregné avec l'acide silicique dans un mélange de diisobutylcétone–acide acétique–eau (40:25:5) leur a permis d'observer la disparition de la lécithine et la formation de lysocithine sous l'influence du venin.

Le fait que, dans les conditions adoptées, la réaction s'avère relativement lente —les observations rapportées concernent l'effet du venin laissé en contact pendant 21 heures à la température ambiante—correspond aux résultats que nous avons précédemment obtenus[22] et qui peuvent être attribués à la différence d'état physique sous lequel se trouve la lécithine du jaune d'oeuf fraîchement diluée et la suspension de lécithines préalablement purifiées.

## RÉSUMÉ

Au cours de l'hydrolyse enzymatique des lécithines la chromatographie sur papier permet de suivre la disparition progressive du substrat, la formation de lysocithine et l'apparition des acides gras libérés.

La concentration de l'enzyme et la vitesse de la réaction ont été appréciées parallèlement par la méthode manométrique de Warburg et par la méthode chromatographique, cette dernière s'étant révélée particulièrement sensible.

Le venin de *Naja-Naja* et le venin d'*Apis mellifica* se révèlent encore actifs à la concentration de $10^{-6}$; la réaction est rapide et l'hydrolyse est pratiquement complète en moins d'une heure lorsqu'on utilise comme substrat la lécithine non purifiée du jaune d'oeuf.

La méthode se prête également à l'étude des inhibiteurs de ces enzymes et les auteurs ont pu mettre en évidence l'action de l'acide 4-aminophénylarsinique.

## SUMMARY

During enzymic hydrolysis of lecithins it is possible, by means of paper chromatography, to follow the gradual disappearance of the substrate, the formation of lysocithin and the appearance of the liberated fatty acids. The concentration of the enzyme and the velocity of the reaction have been estimated both by the manometric method of Warburg and by the chromatographic method, the latter proving to be particularly sensitive.

The venom of *Naja Naja* and *Apis mellifica* were found to be still active at a concentration of $10^{-6}$; the reaction is rapid and the hydrolysis is practically complete in less than one hour if non-purified lecithin of egg yolk is used as substrate.

The method can also be employed to study the inhibitors of these enzymes, of which the authors demonstrated the action of 4-aminophenylarsonic acid.

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# GAS CHROMATOGRAPHY OF OXYGEN-CONTAINING TERPENES

GIAN PAOLO CARTONI AND ARNALDO LIBERTI
*Institute of Analytical Chemistry, University of Messina (Italy)*

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It has been shown by several authors that gas chromatography is one of the more promising tools for the analysis of essential oils. Though several papers have been published on various oils no systematic investigation has been carried out on different classes of compounds.

In a previous paper[1] the behaviour of terpene hydrocarbons in gas chromatography has been described. The purpose of this work is to report the results obtained with more common oxygen-containing terpenes, which are usually found in citrus oils.The technique described should, however, be of value in the study of other terpenes from different sources.

The volatile oxygen-containing terpenes may be classified as alcohols, carbonyl compounds and esters. They have the following properties, which render chromatographic examination rather difficult: high sensitivity to heat and to the presence of foreign substances, which may cause their decomposition, and a high boiling point in quite a small range. Because of these features the working conditions should be chosen very carefully. Suitable working conditions have been established during this work and the retention volumes have been determined on different stationary phases, so that with these values the identification of the nature of an unknown terpene in an essential oil should also be possible.

## EXPERIMENTAL

### *Apparatus*

The chromatographic measurements were made with an apparatus provided with a gas density balance meter as detecting device.

Glass columns of 0.4–0.5 cm internal diameter were employed; they were connected by means of teflon tubing to obtain a total length of 3–5 m.

### *Solid supports*

Various solid supports were tried. Substances such as sterchamol or firebrick gave unsatisfactory results; they do not behave as inert materials, since it was observed that several oxygen-containing terpenes (mainly alcohols and esters) are either strongly retained or decomposed. This is probably due to the presence of metal (iron, aluminum) oxides in these materials.

Celite is an excellent support provided inactivation of the material is carried out correctly. Embacel (May & Baker) has been found quite satisfactory.

### *Stationary liquids*

It was found very convenient to perform the chromatographic separation on columns with quite different properties: one weakly polar, where the components are eluted according to their boiling point and the other strongly polar in order to achieve separations of compounds that can only be separated with difficulty. For the former D.C. 550 Silicone oil was used and for the latter Hyprose S.P. 80, trade name of the Dow Chemical Co. for octakis(2-hydroxypropyl)sucrose. The large number of hydroxyl groups makes this substance particularly suitable for the examination of polar compounds; it has been used up to 160° where its volatility is less than 0.1 %.

The stationary liquids were used in the ratio 20 % w/w. Silicone oil was dissolved in petroleum ether and Hyprose in methanol; after mixing, the solvent was eliminated by gentle heating. The use of low percentages of stationary phases is recommended, in order to obtain low retention volumes; this is one of the main requirements that should be fulfilled, since fairly long columns should be used in order to achieve separation of compounds that boil in a very close range.

A lower percentage of stationary liquid affects the shape of the elution peaks: a 20 % w/w concentration permits operation with a rather high gas flow-rate, which corresponds to a more convenient linear velocity. The best results were obtained with a gas flow-rate of 80–100 ml/min corresponding to a linear velocity of 15 cm/sec. These working conditions give a column efficiency of about 800 theoretical plates per meter of column.

### *Temperature*

Particular attention should be paid to the column temperature; since most terpenes are very sensitive to heat, the columns should be kept at as low a temperature as possible. The compounds that were examined have a boiling range between 180° and 240°; they may be eluted under the conditions described in a fairly short time (60–100 min) by keeping the column temperature between 132°–156°.

These temperatures were obtained by means of boiling cellosolve ($t = 132°$) and cyclohexanol ($t = 156°$). The highest temperature that can be attained without causing decomposition depends on each substance; for instance the decomposition of linalyl acetate, one of the more sensitive esters, is less than 1 % when it is kept for 1 hour at 132°, but it is about 10 % at 156° under the same working conditions.

## RESULTS

Table I shows the retention volumes of several oxygen-containing terpenes; $V$ is the relative retention volume with tetralin (taken as 1) as the reference standard and $V_g$ the retention volume relative to the absolute retention volume of tetralin corrected for the pressure drop and the column temperature; this absolute retention volume was found to be 404 ml/g for silicone oil and 238 ml/g for Hyprose S.P. 80.

In Fig. 1 the logarithm of the retention volume of several oxygen-containing terpenes is plotted against the boiling point; it can be observed that the behaviour of most terpenes is quite different with the two stationary phases used. With silicone oil all terpenes are eluted according to their boiling points, with Hyprose the order

TABLE I

RETENTION VOLUME OF OXYGEN-CONTAINING TERPENES

| Substance | *Silicone D.C. 550* | | *Hyprose S.P. 80* | |
|---|---|---|---|---|
| | $V$ | $V_g$ | $V$ | $V_g$ |
| Tetralin (reference) | 1.00 | 404.0 | 1.00 | 238.0 |
| *Alcohols* | | | | |
| Cineole | 0.38 | 153.5 | 0.33 | 78.5 |
| Linalool | 0.56 | 226.2 | 1.29 | 307.0 |
| Menthol | 0.99 | 400.9 | 2.63 | 626.0 |
| α-Terpineol | 1.10 | 444.4 | 3.37 | 802.0 |
| Citronellol | 1.15 | 464.6 | 4.48 | 1066.2 |
| Nerol | 1.41 | 570.6 | 5.32 | 1266.1 |
| Geraniol | 1.48 | 598.0 | 6.65 | 1583.7 |
| Eugenol | 3.41 | 1378.6 | | |
| *Carbonyl compounds* | | | | |
| Caproic aldehyde | 0.32 | 129.2 | 0.43 | 102.3 |
| Methylheptenone | 0.53 | 214.1 | 0.55 | 131.0 |
| Caprylic aldehyde | 0.57 | 230.2 | 0.67 | 159.4 |
| Citronellal | 0.76 | 307.0 | 1.14 | 271.3 |
| Capric aldehyde | 1.01 | 408.0 | 1.05 | 250.0 |
| β-Citral (Neral | 1.52 | 614.0 | 2.94 | 699.7 |
| α-Citral (Geranial) | 1.85 | 757.4 | 3.63 | 863.9 |
| Carvone | 1.74 | 703.0 | 3.81 | 907.7 |
| Cumic aldehyde | 1.76 | 711.0 | 3.45 | 821.1 |
| Cinnamaldehyde | 2.70 | 1090.8 | 9.98 | 2376.2 |
| *Esters* | | | | |
| Linalyl acetate | 1.34 | 541.3 | 1.24 | 295.1 |
| Geranyl acetate | 2.95 | 1191.8 | 2.98 | 709.2 |

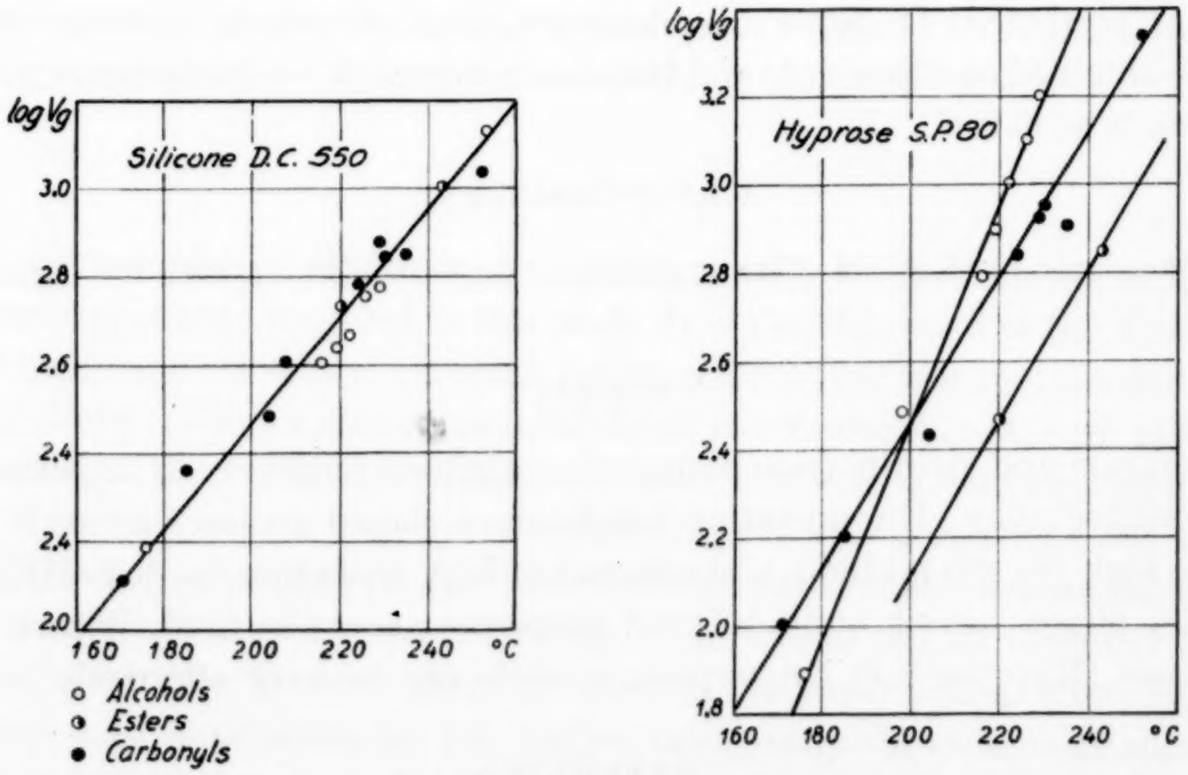

Fig. 1. Log of retention volume *vs.* boiling point for oxygen-containing terpenes on a Silicone column and a Hyprose column.

of elution differs according to the nature of the functional group. The alcohols are more strongly retained than the aldehydes and esters, while terpene hydrocarbons have a very low affinity to Hyprose so that their elution occurs very quickly.

In the analysis of mixtures of terpenes and of essential oils the use of columns with quite different properties is particularly effective for achieving good separations and for the identification of the nature and the functional group of a certain terpene.

In Fig. 2 the logarithm of the retention volume obtained on a Silicone column is plotted *vs.* the logarithm of that on a Hyprose column: with a fair approximation most of the values lie on straight lines.

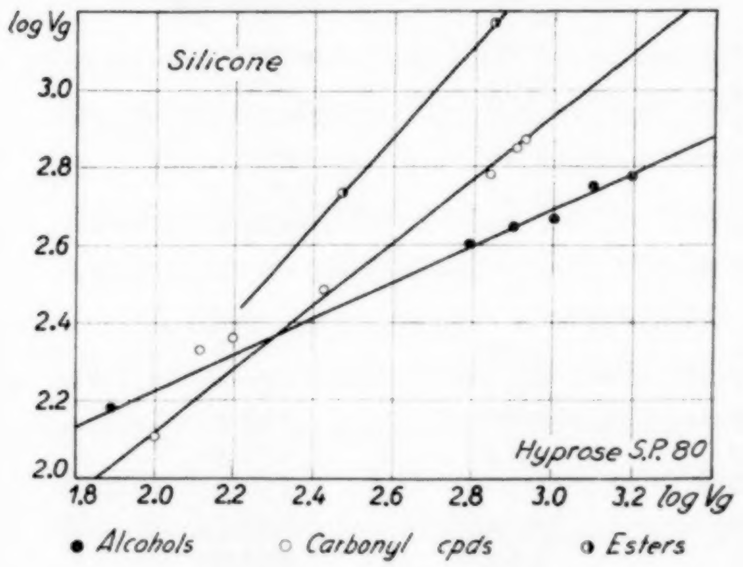

Fig. 2. Determination of the nature of oxygen-containing terpenes by comparing their behaviour on two columns, Silicone D.C. 550 and Hyprose S.P. 80.

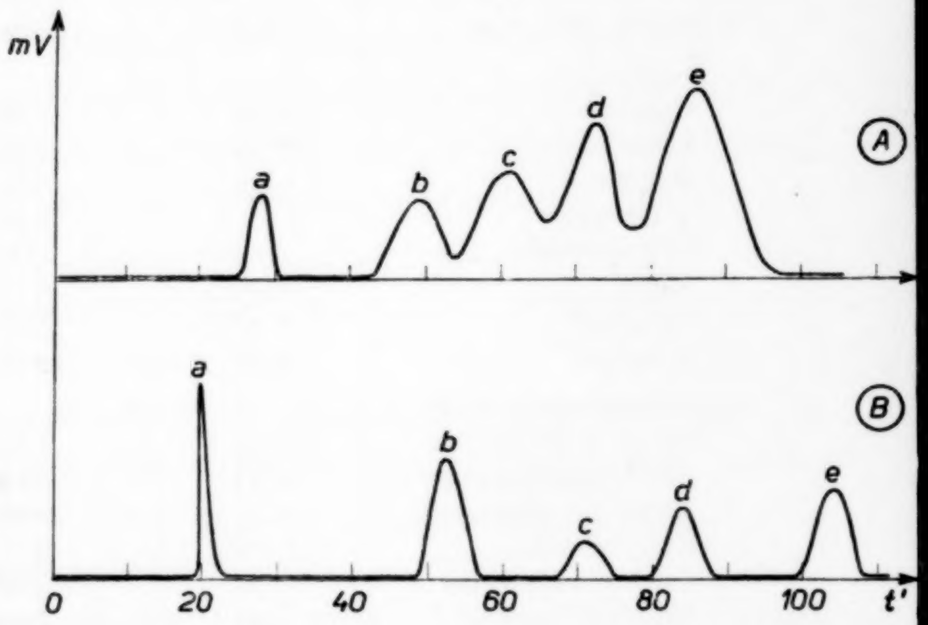

Fig. 3. Gas chromatogram of a mixture of linalool (a), terpineol (b), citronellol (c), nerol (d), and geraniol (e). $t = 132°$, $p = 2$ atm, gas flow-rate 110 ml/min. (A) 3 m column of Celite–Silicone 20%; (B) 3 m column of Celite–Hyprose S.P. 80 20%.

In Fig. 3 one of the more interesting applications of the use of the Hyprose column is presented. It shows the chromatograms of terpene alcohols of similar structure obtained on silicone (A) and Hyprose columns (B); with the latter a complete separation is achieved.

## ACKNOWLEDGEMENT

The authors wish to thank Mr. Giancarlo Cavalle for his help with the experimental work.

## SUMMARY

The separation and identification of oxygen-containing terpenes can be performed by gas chromatography. The operating temperature should be low ($< 160°$), the gas flow-rate high ($\sim 100$ ml/min), and columns of high resolution ($> 3000$ HETP) with stationary phases having quite different properties should be used. With a strongly polar phase (Hyprose S.P. 80) terpene alcohols can be very effectively separated.

## REFERENCE

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# RAPID PAPER IONOPHORESIS USING ORGANIC BUFFERS IN WATER–FORMAMIDE AND WATER–UREA

L. N. WERUM, H. T. GORDON AND W. THORNBURG
*Department of Entomology and Parasitology, University of California, Berkeley,*
*and*
*Biological Research Laboratory, California Packing Corporation, Emeryville, Calif. (U.S.A.)*

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Paper ionophoresis can often separate mixtures not well resolved by paper chromatography, *e.g.*, the 3-methyl and 4-methyl ethers of D-glucose[1]. It can also furnish unique information about the ionizable groups in a molecule, useful in characterizing unknowns. However, it has many limitations. The most serious one is evaporation of water from the paper strip; this can be minimized by using various cooling systems but the apparatus becomes more complex and less convenient to use. Adsorption on the cellulose can distort the ionophoretic movement of spots. Mobility values are usually non-linear with time and not highly reproducible, and ionophoresis is often extremely slow (12 hours or longer).

The work reported in this paper had three main objectives: First, to develop a series of new buffers of various pH values, which would minimize evaporation, adsorption, and non-linear mobility. Second, to develop a series of "tracer dyes", to serve as reference standards for calculation of reproducible relative mobility values. Relative mobility (using glucose as a standard) has been shown to be reproducible and characteristic in ionophoresis of carbohydrates[1]. Third, to reduce the time required for ionophoresis to 3 hours or less, without elaborate apparatus or cooling systems.

## APPARATUS

The Misco micro-analytical ionophoresis apparatus (made by Microchemical Specialties Co., Berkeley, Calif.) was used in this work. It is a modification of the WIELAND-FISCHER design[2], and consists of ten small Lucite cells, 6.5 cm long, 3.5 cm wide, and 3.5 cm high. Each cell has a platinum electrode set in a Lucite mount, with male and female couplings. Five cells are usually set side-by-side in a battery, with their electrodes connected into a common electrode, which is connected to one of the terminals of the DC power supply. The other five cells are similarly connected, and to the other terminal. The two sets are placed 15 cm apart, and an indented Lucite plate (33 × 15 cm) set between them to support the paper strips. Five different buffers can be used simultaneously. The buffers have nearly the same resistance, and all five paper strips operate at the same voltage and current, which remain fairly constant throughout the ionophoresis. The Misco cells are designed for small capacity

(5 or 10 ml), since buffers are used only for a few runs (about 25 mA-hours) and then discarded.

For rapid uniform drying of ionograms, a 9 × 9 inch Misco hot-plate is used. This is an aluminum plate with imbedded heating element controlled by a variable transformer. Very uniform, constant surface temperatures can be obtained by setting the voltage to any desired value. This minimizes spot migration and distortion during drying.

The power supply used need not provide more than 300 V at 30 mA, or have very good voltage regulation. The methods described here cause only minor changes in the resistance of the paper during ionophoresis, and these changes are largely compensated for by the use of relative mobility values instead of absolute mobilities.

## MATERIALS

All the materials used in this work are obtainable from Microchemical Specialties Company, Berkeley, Calif., who also manufacture the apparatus. Most of the materials are also available from various other sources.

Whatman No. 4 filter paper, 1 ½ or 2 inches wide, is available in 600-foot rolls. This is cut into 24-cm lengths. Starting-lines are drawn in pencil at 12 cm and edge-marks at 4.5 and at 19.5 cm; from three to six short cross-lines are drawn intersecting the starting-lines at 6 to 9 mm intervals. Spots are later applied to the points of intersection. This allows only three to six spots to one strip, but greater crowding is not desirable. Special Misco ionophoresis cells are available for paper wider than 2 inches, for preparative work or use when many samples are to be run in the same buffer.

It is possible to arrange a battery of three pairs of Misco cells lengthwise and 30 cm apart, and thereby double the path length available for ionophoretic separation. In our experience this system has proved useless. A superior way of gaining a longer ionophoresis path length is to draw two starting lines, one at 7 cm and one at 17 cm. The unknown solution (and also a spot of ABA reference dye solution) is then spotted (on different lanes) on both starting lines. Cations will be separated on one lane, anions on the other.

### *Buffer solutions*

*FPF, pH 3.3 buffer.* Mix 19 g (16 ml) of 88–90% formic acid and 9 g (8.8 ml) of pyridine. Cool and dilute with water to 100 ml. The stock buffer keeps indefinitely. The dilute buffer is prepared by mixing 2.0 ml of stock buffer, 7.5 ml of formamide, and water to a volume of 25 ml. The dilute buffer is approximately 0.09 $M$ in pyridinium ion, 0.09 $M$ in formate ion, and 0.2 $M$ in formic acid, in 30% (v/v) formamide. If pyridine is objectionable it may be replaced by 10 g of 2-dimethylaminoethanol, or an equimolar amount of some other strong organic base. Anhydrous (98 %) formic acid (Eastman 139 or equivalent) may be used instead of the 90 % acid. The pH of all buffers used in this work falls within 0.05 unit of the intended value, when the stock is diluted with water and measured with a glass electrode.

*DFF, pH 4.0 buffer.* Mix 9 g (7.5 ml) of 88–90% formic acid and 10 g (11.3 ml) of 2-dimethylaminoethanol (Eastman 715 or equivalent). Cool and dilute with water to 100 ml. The stock buffer keeps indefinitely. The dilute buffer is prepared by mixing 2.0 ml of stock buffer, 7.5 ml of formamide, and water to a volume of 25 ml. It is approximately 0.09 *M* in hydroxyethyl-dimethylammonium ion, 0.09 *M* in formate ion, and 0.05 *M* in formic acid.

*ADF, pH 4.7 buffer.* Mix 10 g of 2-dimethylaminoethanol and 13.5 g of glacial acetic acid. Cool and dilute with water to 100 ml. This stock buffer keeps indefinitely. The dilute buffer is prepared by mixing 2 ml of stock buffer, 7.5 ml of formamide, and water to 25 ml. It is approximately 0.09 *M* in hydroxyethyl-dimethylammonium ion, 0.09 *M* in acetate ion, and 0.09 *M* in acetic acid. Other strong organic bases may be used to replace 2-dimethylaminoethanol.

*IAF, pH 6.0 buffer.* Mix 24.6 g $\beta,\beta'$-iminodipropionitrile (Eastman P-6555 or equivalent) and 3.36 g of glacial acetic acid. Cool and dilute with water to 100 ml. The stock buffer keeps for at least six months. The dilute buffer is prepared by mixing 4 ml of stock buffer, 7.5 ml of formamide, and water to 25 ml. The dilute buffer is approximately 0.09 *M* in acetate ion, 0.09 *M* in dicyanoethyl-ammonium ion, and 0.23 *M* in iminodipropionitrile.

*DPF, pH 7.2 buffer.* Mix 19.6 g of 2-dimethylaminopropionitrile (Eastman 6786 or equivalent) and 6 g of glacial acetic acid. Cool and dilute with water to 100 ml. The stock buffer keeps for at least six months, but not indefinitely because of slow hydrolysis of the nitrile to the amide and acid. The dilute buffer is prepared by mixing 3 ml of stock buffer, 7.5 ml of formamide, and water to 25 ml. The dilute buffer is approximately 0.12 *M* in dimethylaminopropionitrile, 0.12 *M* in cyanoethyl-dimethylammonium ion, and 0.12 *M* in acetate ion.

*EAF, pH 8.0 buffer.* Mix 26 g of N-ethylmorpholine (Eastman P-6274 or equivalent), and 6.75 g of glacial acetic acid (plus about 20 ml of water to reduce the violence of the neutralization). Cool and dilute with water to 100 ml. The stock buffer is usually colored, but this does not affect its use. The dilute buffer is prepared by mixing 2 ml of stock buffer, 7.5 ml of formamide, and water to 100 ml. It is approximately 0.09 *M* in acetate ion, ethylmorpholine, and ethylmorpholinium ion. The similar MAF, pH 7.6 buffer is prepared by replacing the N-ethylmorpholine by 22.8 g of N-methylmorpholine. (The use of 2,2′,2″-nitrilotriethanol as a pH 8.0 buffer is not recommended, because it is difficult to remove from the paper strips and interferes with many spray reagents.)

*DAF, pH 9.3 buffer.* Mix 18 g of 2-dimethylaminoethanol (Eastman 715 or equivalent) and 6.6 g of glacial acetic acid. Cool and dilute with water to 100 ml. The stock buffer keeps indefinitely, but slowly becomes yellow in color. The dilute buffer is prepared by mixing 2 ml of stock buffer, 7.5 ml of formamide, and water to 25 ml. The dilute buffer is approximately 0.09 *M* in acetate, 0.09 *M* in hydroxyethyl-dimethylammonium ion, and 0.075 *M* in dimethylaminoethanol.

*DBF, pH 9.3 borate buffer.* Mix 27.8 g of 2-dimethylaminoethanol and 21.9 g of boric acid, and dissolve in water (heating if necessary) to a volume of about 450 ml.

Cool and dilute with water to 500 ml. The stock buffer keeps indefinitely. The dilute buffer is prepared by mixing 17.5 ml of the stock buffer and 7.5 ml of formamide. The dilute buffer is approximately 0.25 *M* in hydroxyethyl-dimethylammonium ion, and 0.2 *M* in dimethylaminoethanol. The composition of the borate–boric acid system is uncertain, but the low conductivity suggests that the predominant form is hydrated tetraborate $(H_3BO_3 \cdot H_2BO_3)_2^{=}$ ion, at 0.125 *M*, with relatively little $H_3BO_3$. The dimethylaminoethanol greatly augments the buffering power, but also increases the viscosity of the buffer.

The pH values of the buffers were determined with a glass electrode, at the recommended dilution *in water, without formamide*. For all the buffers except the pH 7.2 DPF, pH 8.0 EAF, and pH 9.3 DAF, the glass electrode reading *in 30 % formamide* is about 0.5 pH unit *higher*. The three exceptional buffers are tertiary nitrogen bases, and give the same pH reading with or without formamide. The other buffers can form strong hydrogen bonds with formamide, and this increases the p*K* values by about 0.5 unit in 30 % formamide. Such p*K* shifts must be considered when (in a subsequent section of this paper) mobility values are used to calculate the p*K* of unknowns.

As new organic nitrogen compounds become available, especially among the 2-hydroxyethyl, 2-cyanoethyl, cyanomethyl, and 3-hydroxypropyl substitution products of ammonia, it will become possible to prepare superior buffers in the range from pH 5 to 7 and from 7.5 to 9. The IAF (pH 5.9) buffer now uses iminodipropionitrile far from its p*K* (about 5.5–5.6), because no organic buffer with a p*K* near 6 is available. It would be preferable to use pure substances in place of the technical grades indicated above, and all substances should be used, where possible, at the p*K* value (*i.e.*, a 1:1 ratio of the ionized and unionized forms). When finer gradations of pH are required, the ratio can be altered to 1.25:1, 1.6:1, or 2:1, to shift the pH by 0.1, 0.2, or 0.3 units. If the concentration of the ionized form must be increased, however, the ionic strength will be too high. The ratio must therefore be altered by decreasing the concentration of the unionized form, which causes some loss in buffering power.

Since many proteins are denatured even in 10% formamide, organic buffers containing 10% urea are recommended for protein ionophoresis, and the ionic strength must be lowered to one-third that of the formamide buffers. To prepare urea buffers, use one-third of the recommended volume of stock buffer, and use 10 ml of a 25% solution of urea in water instead of 7.5 ml of formamide. The most useful urea buffer is DPU (pH 7.2).

Time may be saved in preparation of buffers (except borate) by using a solution of 30% formamide (or 10% urea) to dilute the stock buffers. The final concentration is somewhat lower, but this usually makes little or no difference.

### *Reference dyes for determination of relative mobility*

The use of glucose as a reference standard of mobility, in ionophoresis in borate buffers, is now widespread, since $M_G$ values are relatively reproducible[1,3]. This principle can be extended to the ionophoresis of all substances by using at least two dyes on each ionogram. One must be a neutral dye having zero charge in all buffers. Another must be an ionized dye whose charge is the same in all buffers. In this work, these two primary standards are the following:

*Amaranth* (FD&C Red No. 2) is the trisodium salt of 1-(4-sulfo-1-naphthylazo)-2-naphthol-3,6-disulfonic acid. Molecular weight of the anion is 523. It is a dark red food coloring, and the commercially available dye is quite pure. At pH values from 3.3 to 9.3, it has three negative charges; the phenolic hydroxyl is undissociated.

*Apolon*, a new common name proposed for a new, slightly water-soluble dye, 4-[4′-(2-hydroxyethyl)-phenylazo]-2″, 2‴-(phenylimino)-diethanol. The name is derived from the Greek *apolos*, meaning "immovable". The dye is synthesized by diazotizing 4-(2′-hydroxyethyl)-aniline and coupling the diazonium salt with 2,2′-(phenylimino)-diethanol. It is a yellow dye, with zero charge in the pH range from 3.3 to 9.3. In all buffers except the borate buffer (DBF) it has nearly the same mobility as the amino acid, proline. In DBF, proline (like all other neutral amino acids) seems to form a weak complex with borate, and moves as if it had a small negative charge relative to Apolon. Preparations of Apolon may also contain an unidentified purple dye, which has a positive charge in the pH range from 3.3 to 9.3 and is easily separated from Apolon during ionophoresis.

On any ionogram, the distance between Apolon and Amaranth (originally spotted together on the starting line) is defined as 100 *Am* units. The mobility of any other substance can be calculated in *Am* units by measuring its distance from the Apolon spot, dividing by the Amaranth–Apolon distance, and multiplying by 100. The charge on the molecule is indicated by a minus sign, if the molecule has moved in the same direction as Amaranth. For example, the mobility of aspartic acid in pH 3.3 FPF buffer is —21, and that of arginine is 68.

This mobility calculation is valid only if the mobility of every substance at every point on the ionogram is constant during ionophoresis. This is approximately true in the center region of the strip (within 25 mm of the center), but mobilities often show a slight decline in the region 25–50 mm from the center. Since the Amaranth spot usually travels in the 25–50 mm region by the end of one hour of ionophoresis, the *Am* value of a slower-moving spot is often larger in a 60-min run than in a 30-min run, since the slow spot moves at a relatively constant velocity, while the Amaranth spot moves more slowly during the second 30 min of ionophoresis. In order to correct for this effect, a second reference dye, moving about half as fast as Amaranth, is introduced.

*Brilliant Blue FCF* (FD&C Blue No. 1) is the disodium salt of 4-{[4-(N-ethyl-*p*-sulfobenzylamino)-phenyl]-(2-sulfoniumphenyl)-methylene}-(N-ethyl-N-*p*-sulfobenzyl)-2,5-cyclohexadien-1-imine. Molecular weight of the anion is 746. It is a deep blue food coloring, and a relatively pure commercial dye. At pH values from 3.3 to 9.3 it has two negative charges, and its mobility in the various buffers is from 50 % to 60 % of that of Amaranth. Standard "*Am*" values have been determined for this dye, using 30-min runs, in all the buffers. For any substance having a mobility equal to or less than that of Brilliant Blue, the *Am* value can be calculated by measuring its distance from the Apolon spot, dividing by the Brilliant Blue–Apolon distance, and multiplying by the standard *Am* value of Brilliant Blue. For substances moving faster than Brilliant Blue, the calculation from the Amaranth–Apolon distance usually gives a more reliable *Am* value.

The reference dyes are combined in one solution (the "ABA" solution) and spotted on each ionogram, and serve as the reference points for calculating the mobility of negatively charged compounds. The purple Apolon-impurity has a relatively low mobility (27 *Am* units) and most positively charged compounds therefore move outside the dye framework. Attempts to find a satisfactory fast-moving, positively charged dye were unsuccessful. Most of the dyes tested lose their charge at pH 9.3, or adsorb very strongly to filter paper, or form insoluble precipitates when mixed with Amaranth. One fairly good dye, used for mobility testing in some of our work, is the well-known antimalarial, quinacrine.

*Quinacrine hydrochloride* (Atabrin, Mepacrine) is 2-methoxy-6-chloro-9-(1'-methyl-4'-diethylaminobutyl)-aminoacridine dihydrochloride. It has two positive charges at pH 3.3 to 7.2, but apparently only one at pH 9.3. It is adsorbed by filter paper, but not as intensely as acriflavin or other dyes with a quaternary nitrogen atom. It has not been used much in this work, although standard *Am* values have been determined. It is usually simpler to use the Brilliant Blue scale for positively charged substances having a mobility equal to or less than Brilliant Blue, and the Amaranth scale for substances of higher mobility. The mobility lag of substances moving in the 25–50 mm region is about the same, on either the anodal or cathodal side of the strip.

## PROCEDURE

(A) Dilute buffers are usually prepared within a few hours before use, by pipetting the proper volume of stock buffer into a 25-ml graduate or volumetric flask, and diluting to the mark with either 30% formamide or 10% urea in water. A 5 ml or a 10 ml aliquot is then pipetted into each of a pair of buffer reservoirs in the Misco apparatus. The apparatus must be properly leveled so that there will be no significant difference in level between the buffer reservoirs; any slight difference merely adds to the electro-osmotic flow, and is corrected for by the neutral dye, Apolon.

(B) A pre-cut 24-cm strip of Whatman No. 4 paper is coated (either with a pipette or a Misco applicator) with the buffer, as uniformly as possible, and slightly undersaturated. The correct volume is about 1.2 ml/100 cm$^2$, or about 1.0–1.1 ml for a strip 24 cm long and 3.8 cm (1.5 inches) wide. One end of the strip is then inserted in one reservoir, the strip is laid across the supporting plate, and the other end inserted in the opposite buffer reservoir. The edge-marks (at 4.5 and 19.5 cm) should be approximately on the edges of the plate. The strip should never be inserted dry and allowed to become saturated by capillary flow from the reservoirs, as the composition and pH of the buffer can be drastically altered (by chromatography) by the time the liquid has reached the center of the strip.

(C) Several strips of paper saturated with distilled water are laid along the walls of the chamber, to humidify the air. The cover is closed, and a low voltage gradient (about 2 V/cm) applied to accelerate equilibration of the paper strips. Within 15 min, any unevenness in coating is smoothed out, and the paper becomes nearly saturated with buffer. The voltage is then turned off and the strips are spotted, usually on the center starting line, but in some cases on lines 50 mm nearer the anode or the cathode (if mixtures only of fast-moving cations or anions are being examined). One of the spots must be the mixture of Amaranth, Brilliant Blue and Apolon, that will serve as mobility reference standard.

(D) After spotting, the cover is closed and about 5 min allowed for mixing of the applied spots with the background buffer. A voltage gradient (usually 10 or 20 V/cm) is then applied, for a period from 30 to 180 min, as required to effect separation. Movement of the reference dyes indicates the degree of separation attained. When fast-moving substances are being studied, ionophoresis is stopped before the Amaranth spot reaches the edge of the strip; with very slow-moving substances, the Amaranth spot is allowed to move off the strip and ionophoresis is stopped when the Brilliant Blue spot nears the edge.

(E) Immediately after turning off the voltage, the ends of the strips are cut off with scissors, leaving only the 15-cm central region. Any long delay in isolating the central region from the buffer reservoirs will allow inflow of buffer into the strip and cause spots that have moved far out from the center to move backward toward the center. During ionophoresis, the central region becomes undersaturated because of evaporation of water from the strip, and backward flow from the reservoirs may displace spots by several mm within 5 min. When the highly saturated ends of the strips are cut off, however, backward flow to the center is negligible.

(F) Each strip is then blotted, by pressing between two strips of filter paper, to remove excess buffer. It is then laid flat on the hot plate for a few minutes, until it is almost dry. When thermolabile substances are being studied, strips can be air-dried overnight. The centers of the reference dye spots can be marked with pencil before drying; they should not be displaced during drying by more than 1 mm. The blotted strips can be extracted with acetone, 2-butanone or ether to remove excess buffer, water, and formamide quickly, without heating. However, it is better to dry the paper strips on the hot plate partially, and then extract. Extraction is highly recommended, because the strips can then be detected with almost any spray reagent and with maximum sensitivity.

(G) The buffer in a pair of reservoirs can be re-used several times during one day, with new strips coated with the excess supply of fresh buffer prepared in the morning. When routine separations are being run, buffer can be used for 2 or 3 days, with as many as 12 successive paper strips. In this case, where precise pH maintenance is not needed, only 6 ml of buffer is pipetted into each reservoir, leaving 13 ml (of the original 25) available for coating strips.

### *Detection of spots on paper ionograms*

Many reagents used for detecting spots on paper chromatograms cannot be used directly on paper ionograms because of residual organic buffer, formamide, or urea. It is usually desirable to extract the ionograms with an organic solvent before detecting spots, but this is sometimes unnecessary because some reagents are specific enough to show no interference from the background buffer. The following reagents have been used in this work, either by dipping the ionograms in the reagent, or by pouring the reagent directly from the stock bottle (or with a pipette) over the ionogram. The "pouring" technique is less satisfactory on unextracted ionograms containing residual formamide, because the formamide may form streaks and distort spots; "pouring" should be used primarily on ionograms extracted or completely air-dried.

*AD-1* is a 0.1 % solution of ninhydrin in acetone, used to detect amines and amino acids. More sensitive ninhydrin reagents have been described[4], but these usually require spraying. Some amino acids develop a blue color when the ionogram is heated over a boiling water or steam bath; other amino acid spots can be developed by careful heating on a hot plate or over a Bunsen flame.

*AD-2* is a 0.1 % solution of alloxan monohydrate in acetone. It is used exactly like AD-1, but is a more sensitive detector for some amino acids.

*CD-3* is a periodic acid–benzidine reagent, previously described for detection of many carbohydrates on paper chromatograms[5]. It can be used without prior extraction to remove buffers, and gives brilliant and sensitive detection on borate ionograms. It is a pouring or dipping reagent, but can also be sprayed if desired. It not only detects polyols but also serine and threonine.

*Bromphenol Blue* (3′,3″,5′,5″-Tetrabromophenol-sulfonephthalein).This is used for detection of proteins that have been heat-denatured by drying ionograms on the hot plate. The ionogram is washed in 5 % (v/v) acetic acid in water to remove buffer and urea, and then immersed in a 0.2 % solution of Bromphenol Blue in 5 % acetic acid for 5 to 10 min. It is then quickly washed in 5 % acetic acid to remove excess dye, in water to remove excess acid, blotted dry and then completely dried (in air, or on the hot plate). Exposure to ammonia vapor then turns the protein spots deep blue. The ionogram can be de-stained by washing in dilute ammonia water, and then re-stained by the above (or some other) procedure, since the protein spots are permanently fixed to the paper.

## RESULTS

### *Osmotic and evaporative flow rates of various buffers*

Since filter paper has some negatively charged groups (probably carboxylic acid) in its structure, application of a voltage gradient to buffer-saturated paper forces the buffer to move as if it were positively charged, toward the anode. All substances in solution are subject to this "electro-osmotic" flow. It can be measured by the displacement of a spot of Apolon applied to the exact center of the paper strip. Values for various buffers and voltage gradients are listed in the column headed "$N_0$" in

TABLE I

OSMOTIC AND EVAPORATIVE FLOW OF BUFFERS ON WHATMAN NO. 4 PAPER AT 20°–22° AT SEVERAL VOLTAGE GRADIENTS

| *Buffer* | | | *V/cm* | *mW/cm²* | $N_0$ | $-N_{+50}$ | $-N_{-50}$ |
|---|---|---|---|---|---|---|---|
| *Type* | *Ionic strength* | *pH* | | | | | |
| FPF | 0.2 | 3.3 | 20 | 34 | 4, 5, —* | 32, 19, —* | 38, 22, —* |
| | | | 10 | 9 | 4, 3, 4 | 4, 6, 5 | 6, 4, 6 |
| | | | 5 | 3 | 2, 2, 2 | 6, 6, 4 | 6, 5, 5 |
| ADF | 0.18 | 4.7 | 20 | 32 | 6, 5, 6 | 42, 29, 21 | 40, 27, 16 |
| | | | 10 | 8 | 6, 5, 5 | 2, 3, 4 | 4, 4, 5 |
| | | | 5 | 3 | 4, 3, 3 | 2, 3, 2 | 2, 2, 3 |
| DPF | 0.24 | 7.2 | 20 | 32 | 10, 6, 6 | 42, 29, 17 | 44, 29, 18 |
| | | | 10 | 8 | 4, 5, 5 | 6, 6, 6 | 6, 6, 6 |
| | | | 5 | 3 | 4, 3, 3 | 4, 4, 3 | 8, 5, 4 |
| DAF | 0.18 | 9.3 | 20 | 32 | 16, 12, 9 | 38, 27, 15 | 40, 30, 18 |
| | | | 10 | 8 | 6, 6, 5 | 4, 4, 5 | 6, 4, 4 |
| | | | 5 | 3 | 4, 3, 3 | 4, 4, 3 | 6, 4, 4 |
| DBF | 0.75 | 9.3 | 20 | 32 | 14, 10, 8 | 42, 27, 16 | 46, 30, 18 |
| | | | 10 | 8 | 10, 9, 8 | 10, 8, 7 | 10, 10, 8 |
| | | | 5 | 3 | 4, 4, 4 | 4, 3, 2 | 6, 5, 4 |

All buffers are the standard 30% formamide systems described under MATERIALS. However, the FPF buffer used in the experiments of Tables I to V had an ionic strength 10% higher than that of the buffer described in the text. This lowered absolute mobility values slightly, but made no other difference. Voltage gradients (V/cm) are approximate, as are values for the electrical heat input in mW/cm².

Spots of the neutral dye (Apolon) were applied in the exact center of the strip ($N_0$), and 50 mm from the center toward the cathode ($N_{-50}$), and toward the anode ($N_{+50}$). All mobilities are in mm/h; three values are given for each spot in sequence. The first value is measured at 30 min, the second at 60, and the third at 120. Low values are not very accurate, since measurements were only to 1 mm. Values for the 50-mm spots have been corrected for osmotic flow by subtracting the corresponding value for $N_0$; *e.g.*, the observed values for FPF at 10 V/cm were 10 and —1 mm/h, which are altered (by subtracting the osmotic flow rate of 4 mm/h) to 6 and —5 mm/h. All values in column $-N_{+50}$ are negative, because spots move toward the anode, "as if" they had a negative charge.

* No value, since paper charred after 1 hour.

Table I. For the pH 3.3 buffer, which partly suppresses the ionization of carboxyl groups, osmotic flow is about 4 mm/h at 10 V/cm. For the pH 4.7, 7.2, and 9.3 buffers it is about 5–6 mm/h; for the pH 9.3 borate buffer, which augments the negative charge on the cellulose by forming weak borate complexes with its glucose units, the rate is 8–9 mm/h. At lower voltage gradients the rate is proportionately less. At 20 V/cm, however, the $N_0$ values in Table I are lower than the theoretical because of evaporative flow. Only five of the buffers listed under MATERIALS have been extensively studied and are included in Tables I to V; tests with the other buffers, however, have given very similar results.

Evaporation of water from the buffer cannot be completely suppressed in the Misco apparatus, although humidifying the air reduces the evaporation rate. Strips through which current is flowing never become much warmer than the ambient air. At low values of electrical heat input, most of the heat is lost by convection, and evaporation is very slow. Since heat input increases as the square of the voltage gradient, there is a critical voltage above which convection cannot dissipate the heat and evaporation then shows a spectacular increase. Data on evaporative flow (in Table I) indicate that this critical voltage gradient is between 10 and 20 V/cm under the conditions used in these experiments. The inflow of buffer from the reservoirs into the center of the strip is very fast at 20 V/cm, of the order of 1 mm/min at a distance of 50 mm from the center. Additional studies (not shown in Table I) indicate that evaporative flow is roughly proportional to distance from the center. At 20 V/cm, it is about 0.5 mm/min at 25 mm from the center, and 2 mm/min at the buffer reservoirs. We can estimate an evaporation rate of about 6 $\mu$l/cm$^2$/h, since the buffer content of saturated Whatman No. 4 paper is about 10 $\mu$l/cm$^2$. This evaporation not only distorts the normal movement of charged substances, but rapidly changes the composition of the buffer. The center of the strip becomes permanently undersaturated, and the buffer in this region may have twice the ionic strength and formamide content of the original buffer. Linearity of voltage gradient is destroyed, and heating of the center may even cause the paper to char.

At 10 V/cm, however, evaporation is less than 1 $\mu$l/cm$^2$/h, and changes in the buffer on the strip are not drastic even after several hours. Nothing is gained by using a lower voltage gradient, since evaporative flow rates are not much less than those at 10 V/cm.

Table II summarizes the results of experiments on varying ionic strength and percent of formamide. Buffers containing 10% urea are similar to those containing 10% formamide. Table II shows that lowering ionic strength to 1/5 of the standard makes it possible to increase the voltage gradient to 20 V/cm without excessive evaporation. This change will seldom be desirable, however, because the lower ionic

TABLE II

EFFECT OF IONIC STRENGTH, FORMAMIDE, AND VOLTAGE ON OSMOTIC FLOW (O.F.) AND EVAPORATIVE FLOW (E.F.)

| *Buffer* | *pH* | *Ionic strength* | *% F or U** | *V/cm* | *mW/cm$^2$* | *O.F. mm/h* | *E.F. mm/h* |
|---|---|---|---|---|---|---|---|
| DA | 9.3 | 0.18 | 30% F | 10 | 8 | 5 | 4–5 |
| DA | 9.3 | 0.036 | 30% F | 20 | 11 | 16 | 9 |
| DA | 9.3 | 0.036 | 30% F | 50 | 80 | 36 | 130 |
| DA | 9.3 | 0.18 | 10% F | 10 | 8 | 4 | 9 |
| DA | 9.3 | 0.036 | 10% F* | 20 | 11 | 14 | 12 |
| DA | 9.3 | 0.18 | 0%* | 10 | 8 | 5 | 10 |
| DA | 9.3 | 0.036 | 10% F | 10 | 2 | 7 | 8 |
| DA | 9.3 | 0.036 | 0%* | 20 | 11 | 10 | 14 |
| DA | 9.3 | 0.036 | 0% | 5 | 0.7 | 3 | 3 |
| FP | 3.3 | 0.04 | 30% F | 20 | 13 | 11 | 12 |
| AD | 4.7 | 0.036 | 30% F | 20 | 11 | 16 | 10 |
| DP | 7.2 | 0.048 | 30% F | 20 | 11 | 15 | 10 |
| DB | 9.3 | 0.15 | 30% F | 20 | 11 | 23 | 26 |

* Spots show cometing at 10% F, streaking at 0%. Effect is more severe at low ionic strength and higher voltage gradients. 10% urea gives slightly higher evaporation than 10% formamide. Values of E.F. higher than 10 usually give unsatisfactory ionograms; values of 5 or less give very good ionograms. Values were determined as in Table I by movement of test spots of Apolon.

strength decreases the capacity of the buffer (spots tending to become larger and less symmetrical) and increases the osmotic flow.

### *Adsorption of substances on the paper strip*

Cellulose adsorbs many substances quite strongly, and this has led many workers to abandon paper ionophoresis in favor of ionophoresis in starch gels or other substrates, especially for separation of proteins. Adsorption can be expressed as a coefficient, $\alpha$, which when multiplied by the "true" or expected mobility of the substance, gives the observed mobility. Adsorption can be measured by saturating a paper strip with a buffer and suspending it from a buffer reservoir so that the liquid flows down the strip by gravity flow. If spots of various substances are applied to a starting line near the top of the strip, all substances should move downward at the same rate unless some are retarded by adsorption. The results of a series of experiments indicate that only the reference dye, Brilliant Blue, is not adsorbed by Whatman No. 4 paper in any of the buffer systems used in this work. Its coefficient may be taken as 1.0 and coefficients for other substances calculated on this basis. Table III gives coefficients for the reference dyes Amaranth and Apolon, and for two smaller molecules (proline and glycerol). The coefficients are altered if the experiment is run in the open air instead of a closed chamber, since evaporation of water lowers the saturation of the paper and increases the actual concentration of buffer and formamide; this usually decreases adsorption.

In all buffers containing 30 % formamide, the coefficients of Apolon, Amaranth, proline, and glycerol are not very different. Omission of the formamide increases the adsorption of the dyes but decreases that of proline and glycerol. Apolon is not a perfect zero-mobility indicator in the aqueous or 10 % urea buffers, if there is any large displacement by evaporative or osmotic flow. This will usually be obvious since the Apolon spot will streak badly. The reduction of strong adsorptive effects by formamide is a very useful characteristic of the buffers. Solvation by formamide may tend to equalize the attractive forces between molecules of different size and chemical structure and molecules of cellulose. Urea has a similar action, but cannot be used at a high concentration.

The most deleterious effect of adsorption is not the reduction in mobility, but the very marked tailing and streaking of spots, which makes it difficult to locate the center of a spot and which may cause adjacent spots to overlap.

The problem of adsorption is discussed in an unusually thorough way by McDONALD[6], who emphasizes the importance of saturation or "wetness" of the paper, of the structure, charge, and concentration of the moving substance, and of the type of paper used. All these factors make the adsorption coefficient unpredictable, and it must be determined empirically.

### *Mobility of reference dyes*

Table IV gives mobilities (in mm/h, relative to Apolon) of Quinacrine (Q), Brilliant Blue (B), and Amaranth (A) in various buffers, at various voltage gradients, and with

## TABLE III

ADSORPTION COEFFICIENTS FOR REFERENCE DYES IN VARIOUS BUFFERS ON WHATMAN NO. 4 PAPER
(relative to Brilliant Blue, taken as 1.0)

| *Substance* | *FPF* | *PFU* | *PF* | *ADF* | *ADU* | *AD* | *DPF* | *DPU* | *DP* | *DAF* | *DAU* | *DA* | *DBF* | *DBU* | *DB* | $H_2O$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Apolon | 0.83 | 0.61 | 0.52 | 0.87 | 0.54 | 0.36 | 0.88 | 0.55 | 0.41 | 0.87 | 0.50 | 0.40 | 0.81 | 0.67 | 0.63 | 0.70 |
| Amaranth | 0.89 | 0.77 | 0.67 | 0.90 | 0.78 | 0.65 | 0.93 | 0.82 | 0.64 | 0.91 | 0.74 | 0.64 | 0.91 | 0.82 | 0.82 | 0.90 |
| Proline | 0.91 | 0.99 | 1.02 | 0.87 | 0.88 | 1.00 | 0.88 | 0.95 | 0.97 | 0.86 | 0.95 | 0.95 | 0.86 | 0.92 | 0.91 | 1.03 |
| Glycerol | 0.94 | 1.00 | 1.05 | 0.90 | 0.91 | 1.00 | 0.84 | 0.91 | 0.97 | 0.89 | 0.92 | 0.95 | 0.85 | 0.92 | 0.90 | 1.00 |

## TABLE IV

MOVEMENT (mm/h) OF REFERENCE DYES AT SEVERAL VOLTAGE GRADIENTS

| *Buffer* | *V/cm* | *Q* | $Q_{+50}$ | $-B_0$ | $-B_{-50}$ | $-A_0$ | $-A_{-50}$ | $N_0$ | *Standard Am values* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | *Q* | *B* |
| FPF (pH 3.3) | 20 | 60, 40, —* | 62, 40, — | 56, 46, — | 50, 47, — | 81, 61, — | 84, 67, — | 5 | | |
| | 10 | 28, 33, 35 | 30, 35, 34 | 28, 30, 32 | 28, 31, 29 | 42, 49, — | 42, 47, 48 | 4 | 71 | 63 |
| | 5 | 16, 16, 16 | 16, 15, 16 | 14, 14, 14 | 14, 15, 13 | 22, 22, 22 | 20, 22, 21 | 2 | | |
| PFU | 10 | | | 36, 30, 25 | | 36, 35, 31 | | 5 | — | (82) |
| PF | 10 | | | 34, 31 | | 28, 28 | | 6 | — | — |
| ADF (pH 4.7) | 20 | 58, 43, — | 56, 38, 32 | 40, 30, 32 | 44, 31, 31 | 76, 58, — | 78, 59, — | 6 | | |
| | 10 | 28, 33, 31 | 28, 34, 32 | 24, 27, 24 | 26, 27, 25 | 44, 47, 43 | 40, 47, 44 | 5 | 70 | 57 |
| | 5 | 14, 15, 15 | 17, 18, 18 | 12, 12, 12 | 12, 13, 13 | 22, 22, 22 | 22, 22, 23 | 3 | | |
| ADU | 10 | | | 34, 29, 26 | | 40, 39, 37 | | 4 | — | (72) |
| AD | 10 | | | 36, 27 | | 30, 26 | | 4 | — | — |

(Continued on p. 137)

TABLE IV *(continued)*

| *Buffer* | *V/cm* | *Q* | $Q_{+50}$ | $-B_0$ | $-B_{-50}$ | $-A_0$ | $-A_{-50}$ | $N_0$ | *Standard Am values* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | *Q* | *B* |
| DPF (pH 7.2) | 20 | 56, 45, — | 52, 38, 39 | 42, 32, 32 | 46, 33, 36 | 80, 63, — | 80, 64, 49 | 6 | | |
| | 10 | 32, 29, 27 | 28, 29, 27 | 22, 25, 23 | 24, 26, 23 | 40, 43, 44 | 40, 45, 41 | 5 | 66 | 57 |
| | 5 | 14, 16, 15 | 16, 14, 14 | 14, 13, 12 | 12, 14, 13 | 24, 23, 21 | 22, 23, 22 | 3 | | |
| DPU | 10 | | | 30, 26, 21 | | 40, 37, 34 | | 5 | — | (68) |
| DP | 10 | | | 34, 25 | | 30, 28 | | 4 | — | — |
| DAF (pH 9.3) | 20 | 34, 26, 30 | 34, 24, 30 | 48, 35, 30 | 50, 34, 29 | 90, 69, — | 88, 65, 55 | 12 | | |
| | 10 | 16, 17, 17 | 16, 17, 15 | 28, 29, 26 | 24, 29, 27 | 46, 50, — | 44, 50, 49 | 6 | 34 | 57 |
| | 5 | 8, 8, 8 | 10, 9, 8 | 16, 15, 14 | 14, 15, 13 | 26, 26, 25 | 26, 26, 25 | 3 | | |
| DAU | 10 | | | 34, 30, 27 | | 44, 42, 43 | | 4 | — | (70) |
| DA | 10 | | | 36, 30 | | 32, 32 | | 4 | — | — |
| DBF (pH 9.3) | 20 | 16, 16, 19 | 14, 16, 18 | 32, 24, 21 | 32, 23, 21 | 66, 48, 41 | 62, 46, 40 | 12 | | |
| | 10 | 10, 8, 10, 10 | 8, 8, 8, 9 | 24, 21, 19, 16 | 24, 21, 19, 18 | 46, 40, 37, 34 | 44, 40, 36, 32 | 8 | 27 | 50 |
| | 5 | 4, 4, 4 | 4, 4, 5 | 8, 9, 9 | 10, 11, 9 | 18, 18, 18 | 20, 20, 18 | 4 | | |
| DBU | 10 | | | 24, 17, 10 | | 38, 30, 21 | | 5 | — | (55) |
| DB | 10 | | | 18, 12 | | 30, 22 | | 7 | — | (55) |

Dye spots were applied in the exact center of the strip (*i.e.* $Q_0$), and 50 mm from the center toward the cathode (*i.e.* $B_{-50}$), and toward the anode (*i.e.* $Q_{+50}$).
First number in column under each dye refers to mm/h movement after first 30 min, second number after 1 h, third number after 2 h, fourth number after 3 h.
Numbers in last two columns refer to calculated *Am* values for Quinacrine (*Q*) and Brilliant Blue (*B*) dyes, and are the ratios of *Q* or *B* movement to Amaranth movement × 100.

* No value, since spot moved off paper.

initial spots applied either at the center (indicated by subscript zero, *e.g.*, $Q_0$) or 50 mm from the center of the strip (indicated by subscripts of +50 if toward the anode, and —50 if toward the cathode). Values for the first 30 min of ionophoresis are relatively inaccurate because centers of spots were not located to better than ± 1 mm, and movement was relatively small (less than 25 mm in most cases). During the initial period, also, some time is required for establishing a relatively steady state.

During the first 30 min, absolute mobility is nearly proportional to voltage gradient, even at 20 V/cm (except for the very high ionic strength DBF buffer). The location of the initial spot (at 0 or at 50 mm) usually does not affect the mobility value.

After one hour, however, mobilities at 20 V/cm are only from 1.1 to 1.5 times those at 10 V/cm, since the effect of the strong evaporative flow is to drive all spots toward the center of the strip. A gradient of 20 V/cm should never be used when a reliable mobility value (for characterization of a compound) is required. In a few instances, the use of 20 V/cm gives good separation of mixtures in 50 % less time than that required at 10 V/cm, and may therefore be advisable.

At 10 V/cm, ionophoretic mobilities are nearly constant even after 2 h, at all points on the paper strip. There is an approximately 10 % decline from the one-hour value in most cases, but this is largely corrected for if mobilities are expressed in *Am* units rather than in mm/h.

For each of the standard buffers, standard values for mobility (in Amaranth units) can be calculated from the mobilities (at 1 and 2 h) at 10 and 5 V/cm. The value for Brilliant Blue is constant at 57 at pH 4.7 to 9.3, but is somewhat higher at pH 3.3 and somewhat lower in pH 9.3 borate. The small changes probably reflect minor interactions with pyridinium or hydrogen ions and with borate ions. For Quinacrine, the *Am* value is about 70 at pH 3.3 and 4.7, declines slightly at pH 7.2 as the p*K* of the acridinium ion is neared, and falls to half the acid value at pH 9.3, where the acridine ring nitrogen is probably uncharged, while the nitrogen of the dialkylamino group retains its positive charge. There is a further slight decline in pH 9.3 borate, possibly indicating weak complexing with borate ions.

In buffers containing 10 % urea instead of 30 % formamide, the *Am* values for Brilliant Blue are higher. The value is only 10 % higher in 9.3 borate, where the high ionic strength exerts a strong anti-adsorptive effect, but it is 20 % higher at pH 9.3 to 4.7, and 30 % higher at pH 3.3. In buffers containing neither formamide nor urea, Brilliant Blue moves nearly as fast or faster than Amaranth (except in the borate buffer). This effect is largely due to a striking fall in the absolute mobility of Amaranth, since the mobility of Brilliant Blue is almost unaffected by formamide or urea, and is largely explained by the strong adsorption of Amaranth at low formamide (or urea) concentration. It is not necessary to abandon "Amaranth units" as standard mobility scale, however. All that need be done is to use Brilliant Blue as the reference dye (in systems containing less than 30 % formamide), and use the standard *Am* values obtained for Brilliant Blue in 30 % formamide buffers.

All methods of paper ionophoresis other than those described in this paper will probably give stronger adsorption effects, and mobility values will often differ from those obtained in this work. If other workers wish to use reference dyes with buffers not containing formamide, we recommend that a mixture of Apolon and Brilliant Blue be used, with the distance between the two dyes defined as 60 *Am* units.

### *Separation and characterization of small organic molecules*

Table V gives mobilities (in Amaranth units) for a few amino acids, peptides, and carbohydrates in the standard (30% formamide) buffers at 10 V/cm. All except borate were run for 60 to 120 min; borate runs were 150 to 180 min. Spots were 1 µl, concentrations 0.02 *M* to 0.1 *M*. Carbohydrates and amino acids were run both singly and in mixtures; *Am* values were the same. When a substance was known or expected to have a low mobility in a buffer, it usually was not run; this accounts for many of the blank spaces in the table.

TABLE V

MOBILITIES (IN AMARANTH UNITS) OF SOME SMALL ORGANIC MOLECULES*

| *Substance* | *FPF*<br>*pH 3.3* | *ADF*<br>*pH 4.7* | *DPF*<br>*pH 7.2* | *DAF*<br>*pH 9.3* | *DBF*<br>*pH 9.3* |
|---|---|---|---|---|---|
| Serine | — | — | — | —28 | —51 |
| O-Phosphoserine | —60 | —66 | —74 | —95 | —90 |
| Glycine | 7 | 0 | 0 | —12 | —37 |
| O-Phosphoethanolamine | — 1 | —10 | —33 | —57 | —58 |
| Alanine | 5 | — | — | —10 | —22 |
| β-Alanine | 61 | — | — | — 3 | —15 |
| γ-Aminobutyric acid | 74 | 13 | 2 | 0 | —17 |
| Proline | 0 | 0 | 0 | 0 | — 8 |
| Aspartic acid | —21 | —71 | —76 | —81 | —82 |
| Glutamic acid | — 7 | —60 | —72 | —77 | —77 |
| Glycyl-glycine | 33 | 1 | — 2 | —73 | —80 |
| Glycyl-asparagine | 22 | 1 | — 2 | —58 | —65 |
| Glycyl-alanine | — | — | — | —72 | —76 |
| Alanyl-glycine | — | — | — | —66 | —70 |
| Glycyl-glycyl-glycine | 30 | — | — | —64 | —66 |
| Histidine | 68 | 63 | 8 | —10 | —33 |
| Arginine | 68 | 68 | 62 | 45 | 33 |
| Sucrose | — | — | — | 0 | —16 |
| Lactose | — | — | — | 0 | —33 |
| Mannose | — | — | — | 0 | —48 |
| Glucose | — | — | — | 0 | —64 |

* Ionophoresis for 1 or 2 h at 10 V/cm, except borate (DBF) buffer which was run for 2 to 3 h. One Amaranth unit corresponds to an absolute mobility (relative to Apolon) of about 0.3 mm/h in DBF, and about 0.45 mm/h in the other buffers. Two spots are clearly resolved if their mobilities differ by about 8 *Am* units.

The results for the amino acids are essentially those predictable from the p*K* values of the ionizable groups. The most useful single buffer for characterizing an unknown is probably the FPF pH 3.3 buffer; if *Am* values in this buffer and also in the DAF pH 9.3 buffer are determined, an unknown can often be identified at least as to its class (*i.e.*, as a basic, neutral, or acidic simple peptide or amino acid).

The classic paper of CONSDEN, GORDON AND MARTIN[8] gives a mathematical analysis of the optimal pH for separation of substances whose p$K$ values and ionic mobilities are very similar; the optimal pH lies between the two p$K$ values, and somewhat closer to the p$K$ of the substance having the lower ionic mobility. For aspartic and glutamic acids (p$K$'s 3.65 and 4.25), the optimal pH is about 4.0, since glutamic acid has a slightly lower mobility than aspartic acid. Difficult separations may require very precise control of the buffer pH.

The data in Table V may be used to calculate approximate p$K$ values of ionizing groups. If we assume that the decline in mobility of histidine from pH 3.3 to pH 7.2 is due to loss of one proton, and that the *Am* value of 68 at pH 3.3 is largely due to this one positive charge, the ratio of the acidic form to the basic form at pH 4.7 is 63/5 and at pH 7.2 it is 8/60. By adding the logarithm of this ratio to the pH of the buffer we obtain estimated p$K$ values of 5.8 and 6.1 (correct value 6.0 for imidazole group of histidine[7]). Analogous calculations for the acidic group of aspartic acid give ratios of 5/71 at pH 4.7 and 55/21 at pH 3.3, and estimated p$K$ is 3.6 and 3.7 (correct value 3.65). For glutamic acid, the ratios are 12/60 and 65/7, and estimated p$K$ is 4.1 and 4.2 (correct value 4.25).

Calculated p$K$ values agree with the correct values when based on ionophoretic mobilities in the acidic buffers, but not in the three alkaline buffers containing tertiary nitrogen bases. For example, values for the alpha-amino group p$K$, if calculated from mobilities in Table V for the amino acids in the pH 9.3 DAF buffer, are all approximately 0.5 pH unit higher than the correct value. The reason is the "formamide p$K$-shift" noted in the description of the buffers. All the ionizing groups of the amino acids can form hydrogen bonds, and their p$K$ values are therefore increased by 0.5 pH unit in 30 % formamide. In the acidic buffers (which are "hydrogen-bonding"), the p$K$ of the buffer also shifts and the effects cancel out. In the alkaline (and "non-hydrogen-bonding") buffers, only the amino acids undergo the p$K$-shift. Therefore, when the p$K$ of an unknown is calculated from mobility values in the "non-hydrogen-bonding" buffers, it will usually be desirable to *subtract* 0.5 pH unit from the estimated p$K$ value.

Table V indicates that one charged group confers a mobility of about 65 to 70 *Am* units to molecules of molecular weight about 150 (histidine, arginine, aspartic acid, glutamic acid, glycyl-glycine). For $\gamma$-aminobutyric acid (molecular weight 103) the maximum *Am* value per charge is probably 75 to 80, and for $\beta$-alanine (molecular weight 89) it is probably 85 to 90. If we assume a value of 90 for $\beta$-alanine, the ratio of acidic to basic form at pH 3.3 is about 62/28, and the p$K$ of its carboxyl group is therefore 3.6 (correct value, 3.6). It is clear that a series of *Am* values at different pH's will sometimes make it possible to estimate either the p$K$, or the molecular weight, or sometimes both.

Amaranth and Brilliant Blue have molecular weights of 500–700 and an *Am* value per negative charge of about 33. It is probable that very large molecules (like proteins) have much lower *Am* values per charge. The empirical value $800/\sqrt{M}$ roughly predicts the *Am* value per charge as a function of the molecular weight, $M$.

The data of WEBER[9] (for a large series of amines in pH 3.8 citrate buffer) suggest that the *Am* value per charge would be about 150 for propylamine, 200 for methylamine, and 250 for very small ions like ammonium or potassium.

### *Mobility of borate complexes*

The DBF buffer is a "hydrogen-bonding" buffer, and gives mobilities for most of the amino acids that agree well with those predictable from the known p*K* values of the $\alpha$-amino groups. This is presumably due to the mutual cancellation of the formamide p*K*-shifts, as in the acidic hydrogen-bonding buffers. However, calculated p*K* values for the $\alpha$-amino group of aspartic and glutamic acids are 0.3 pH unit too high, and for the imino group of proline 0.3 pH unit too low. It is possible that interactions with borate (or other buffers) may sometimes alter the p*K* values (or the molecular size and shape and charge), so that the p*K* calculated from mobility will not be identical with that obtained in dilute aqueous solution. It is important to note that an increased negative charge on an unknown in the pH 9.3 DBF buffer, compared to that in the pH 9.3 DAF buffer, does not always prove that borate-complexing groups are present in the molecule, especially if the molecule has a hydrogen-bonding ionizing group whose p*K* is in the region from pH 8.5 to 10.5.

In the separation of carbohydrates, DBF gives results similar to those obtained by other workers with borate buffers. Expected *Am* values for sugars can be calculated from the data in Table X of the review by MICHL[3], which gives mobilities relative to glucose. The expected *Am* value for mannose is —47, for lactose —25, and for sucrose —12. The latter two values are about 25 % lower than those in Table V. This may in part reflect a difference between the mobility of Apolon and that of the zero mobility standard used in previous studies (usually proline or a methyl-substituted glucose). It may also be a result of the very high borate concentration of DBF (nearly four times higher than that used by other workers), and the very powerful additional buffering provided by the dimethylaminoethanol in DBF, both of which will intensify complex formation. CONSDEN AND STANIER[10] observed that use of a "strong" borate buffer increased the mobilities of many carbohydrates (but not of ketoses or ribose) by 30 %, when compared to their usual, weak buffer. The absolute mobility of glucose in DBF is only $5 \times 10^{-5}$ cm$^2$/volt-second, compared to the value of $14.5 \times 10^{-5}$ reported by CONSDEN AND STANIER. The discrepancy is probably due to the much higher ionic strength and viscosity of DBF. The ionophoretic system used by CONSDEN AND STANIER, however, probably had higher osmotic flow rates and more intense paper adsorption effects than those obtained with DBF. Both relative and absolute mobility values in borate buffers are dependent on borate concentration, pH, buffer viscosity (and therefore temperature), adsorptive power and resistance to liquid flow of the paper used, and even the quantity of the carbohydrate spotted. Relative mobility values in any one ionophoretic system are fairly reproducible, but there will be differences in the values obtained in different systems. Rough tests on many carbohydrates in our system indicate that mobilities relative to glucose agree fairly closely with the values tabulated by MICHL[3], except that our mobility values below $M_G$ 0.50 are often somewhat higher.

The structure of borate complexes is not definitely known. FOSTER[1] suggests a "monodentate" complex and a "bidentate" complex, in which one negatively charged boron atom forms a chelate ring with one molecule or two molecules of polyol. MICHL[3] suggests that a "tridentate" complex may be possible with certain cyclitols, but the evidence is not conclusive. The data of FOSTER and his co-workers, tabulated by MICHL[3], on the mobility of various substituted glucoses prove that glucose has several reactive configurations. The most active configuration (A) is apparently that of the hydroxyls on carbons 2 and 4 in the open-chain (aldehydo) form. The configuration (B) of the hydroxyls on carbons 1 and 2 in the cyclic form also has a high affinity for borate. Several other configurations react with borate, but much more weakly. We conclude that it is likely that most of the complexed glucose (at pH 9.3 and high borate/glucose ratio) is in a 1:1 borate complex either as A or B, with low concentrations of other types of complexes. The *Am* value of glucose is —65, which is a reasonable value for a complex with one charge and a molecular weight less than 200; a 2:1 complex, with a molecular weight of more than 360, should have a much lower mobility. It is remarkable that no carbohydrate has a higher mobility than glucose; this suggests that nearly 100 % of the glucose in a spot is in a borate complex. Substances with configurations having a lower affinity for borate, such as mannose, are presumably partly uncomplexed and partly in 2:1 complexes, and so have lower mobilities. Most pentitols and hexitols have mobilities near that of glucose, but glycerol has a very low mobility ($M_G$ 0.44, or about 30 *Am* units). This suggests that only linkages between borate and two *secondary* hydroxyl groups in a molecule (especially the *β-cis*-configuration noted by FOSTER[1]) are very strong. Glycerol cannot form such linkages, and is probably only partly complexed (largely as a 2:1 complex). The unusually high mobility of the all-*cis* isomers of inositol and quercitol[3] ($M_G$ 1.60, or about 100 *Am* units) suggests that two borate ions may complex with one molecule.

Mixed complexes between a molecule of carbohydrate and the cellulose of the paper may be largely responsible for the lowering of mobility by adsorption. Presumably, two different carbohydrate molecules in a mixture may similarly interfere with each other, so that mobility values will not be the same as those obtained in the ionophoresis of pure substances. This interference will probably be most severe between substances of relatively low and nearly equal mobility, since the percentages of uncomplexed forms will be high and separation will be slow.

### *Resolving power in ionophoresis*

In the ionophoresis of mixtures, with a starting spot of 1 μl volume, spot radius is 2–3 mm. Mobilities must therefore differ by about 6 mm/h (equal to 12 *Am* units) for clear separation in a 1-h run. In the 2- or 3-h runs required for separation of slow-moving spots, a mobility difference of 4–6 *Am* units should be sufficient, but the spreading of spots by diffusion blurs the separation if the difference is less than 8 *Am* units. In the DBF borate buffer (which gives lower absolute mobilities), a difference of 10–15 *Am* units may be necessary for complete resolution. In buffers containing less than 30% formamide, adsorption on cellulose may cause streaking or tailing of spots and lessen the resolving power. If the ionic strength of the solution spotted is much higher than that of the background buffer, spots become very large and even a difference of 20 *Am* units may not give clear separation. If the solution spotted causes a local pH shift in the background buffer, resolution of spots may be lessened. This is why it is desirable to use buffers at a high ionic strength and at a pH near the p*K*, where buffering power is maximal.

### *Ionophoresis of proteins*

When used for separation of human plasma proteins, the 0.06 ionic strength, 10% urea buffers give very fast separations at gradients of 10 to 15 V/cm, in 30 to 120 min. Four or five protein spots can usually be obtained. Albumin has a mobility of about —45 *Am* units (relative to Brilliant Blue, taken as —57 *Am* units) at pH 9.3, —30 *Am* units at pH 7.2, and 37 *Am* units at pH 3.3. The clearest ionograms are at pH 7.2, where adsorption and denaturation effects seem to be minimal. There seems to be no advantage to the use of lower voltage gradients and longer ionophoresis time, since diffusion and adsorption spread the protein spots. Operation of the apparatus in a cold room, however, would probably be advantageous.

If the 10% urea is omitted, similar but somewhat less clear ionograms are obtained. Ionograms may be dried by immersion in cold acetone, to preserve enzymes that would be destroyed by the usual hot-plate drying step.

### *Advantages and disadvantages of the organic buffers*

The major reason for using organic ions is that their conductance (at any given ionic strength) is lower than that of the inorganic ions commonly used, and it is possible to use both high ionic strength and high voltage gradients without excessive heating and evaporative flow. High ionic strength is a great advantage in the ionophoresis of unknown solutions containing relatively high salt concentrations. Spots move compactly, without streaking, even when heavy loads (ionic strength 0.2) must be used (either because the method of detection is insensitive, or because the substance being sought is a minor component).

The gain in mobility made possible by higher voltage gradient is partly nullified by the higher viscosity of the buffers, so that absolute mobilities are usually lower than those obtained by other workers. The high ionic strength also depresses absolute mobility; McDONALD[6] has shown that mobility is inversely proportional to the square root of the ionic strength. We have roughly confirmed this rule, since our 30% formamide buffers used at about 20% of the standard ionic strength give for Amaranth and Brilliant Blue absolute mobility values that are 2.4 times those at the standard ionic strength. Electro-osmotic flow is also about 2.4 times faster.

When the ionic strength of the solution being spotted is 0.05 or less, it may be advantageous to lower the ionic strength of the organic buffers by a factor of 2 to 4, so that separation in a given time will be approximately doubled.

All of the organic buffers except borate can be easily removed from the paper by washing with acetone or 2-butanone (which may be acidified with acetic acid), without removing from the paper spots of many polar organic compounds (such as amino acids, peptides, or carbohydrates). This makes it convenient to elute bands from ionograms for further study, after removal of the buffer.

The major disadvantage of the organic buffers is possible interference with the detection of spots on the ionogram. Many spray reagents are specific, and give no background color with the organic buffers. If a non-specific reagent must be used, it may be possible to extract out the organic buffer with acetone before spraying. The use of different organic buffers may make possible the use of some reagents not compatible with those devised for our researches. The present work attempts to keep the number of organic buffers at a minimum. This is why dimethylaminoethanol, which is primarily useful because its p*K* is 9.3, is also used in the pH 4.7 acetate buffer, although other organic cations might be preferable.

The 30% formamide in the standard buffers minimizes evaporative flow at a gradient of 10 V/cm, and makes possible fast ionophoresis without the use of cooling systems. It also has a very desirable anti-adsorptive effect, which increases the mobility of many substances and (even more important) prevents spots from tailing and streaking. Its disadvantage is low volatility, which requires high, uniform heating to dry the paper without distortion of the pattern of spots. A minor disadvantage is that buffers must be prepared daily from stock solutions, to minimize the hydrolysis of formamide.

The 10% urea remains on the paper on drying and may interfere with detection by some spray reagents. It is chiefly useful for protein separations. It can be easily removed from the paper by washing with methanol or ethanol, which does not remove most proteins.

Buffers more acid than pH 3 or more alkaline than pH 10 cannot be used with 30% formamide, because hydrolysis of the formamide causes the pH to shift rapidly to the 3–10 range. Dimethylformamide is somewhat more stable, and can be used even at pH 2; however, slow hydrolysis causes some pH drift even in 1 h, and such buffers can only be used within an hour after preparation. Dimethylformamide is more volatile than formamide, and is much less effective in preventing evaporation at gradients of 10 V/cm; it must be used at percentages higher than 30% or at lower voltage gradients. Nevertheless, it may sometimes prove useful because it is a good anti-adsorptive agent and its volatility makes it easy to remove from the paper by air-drying.

## ACKNOWLEDGEMENTS

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## SUMMARY

Organic buffers of high ionic strength but low conductance, in 30% formamide, are useful for rapid paper ionophoresis in the pH range 3.3 to 9.3. The apparatus is designed for simultaneous use of as many as five different buffers. Charged substances move as compact spots, without adsorption on paper, and with constant mobility in runs lasting 1 to 3 hours. Mobilities are measured relative to a set of reference dyes. From mobility values it is often possible to estimate the molecular weight of an unknown, the p*K* value of some acidic or basic groups, and the presence of borate-complexing groups. The buffers are useful in separation and characterization of amino acids, peptides, and carbohydrates. If formamide is replaced by 10% urea, rapid separation of proteins can be effected.

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# CHROMATOGRAPHIC STUDY OF ANIONIC COMPLEXES

## PART IV. SEPARATION OF SOME IONS IN THE PRESENCE OF TARTRATE, USING ETHANOL AS SOLVENT

ERIC JOHN SINGH AND ARUN K. DEY
*Chemical Laboratories, University of Allahabad (India)*

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Complex formation plays an important role in the separation of ions in chromatography, as pointed out by POLLARD and coworkers[1,2], who observed that for the separation of metal ions on filter paper the formation of complexes is essential. Various workers, including BURSTALL *et al.*[3] and LEDERER[4], have studied the separation of inorganic ions by paper chromatography, using different solvent mixtures with or without complexing agents. In several earlier papers[5–8] we have described our studies on the diffusion of complex ions formed by the interaction of metal ions with the carboxylic acid groups of oxalic, tartaric and citric acid. In these studies filter paper chromatography was used with aqueous ethanol as solvent. We have also investigated how the $R_F$ values of the ions formed in mixtures vary according to the proportion of the metal ion and the complexing agent used, with a view to determining the effect of varying concentrations of the complexing agent. In this paper we have extended the work to some mixtures of ions and have attempted to employ complex-formation with tartrate for the separation of ions by filter paper strip chromatography, using aqueous ethanol as solvent.

Mixtures of copper(II), nickel(II), cobalt(II) and cadmium(II) were investigated and the results obtained in a large number of experiments are summarised here.

### EXPERIMENTAL

Solutions of cupric sulphate, nickel sulphate, cobalt sulphate and cadmium chloride were prepared from BDH Analar grade chemicals and standardised as usual. A standard stock solution of sodium tartrate was prepared and diluted as required. All other reagents used were of reagent quality. Strips of Whatman filter paper No. 1 were used for the chromatograms. A simple ascending filter paper strip method, as described by GAGE, DOUGLASS AND WENDER[9], was employed. Various concentrations of aqueous ethanol were tried and it was found that in most cases 50% ethanol was suitable as solvent. A series of mixtures was prepared in which the concentrations of the metals were the same, but the concentration of tartrate varied, while the total volume of each solution was kept constant. For the metal to tartrate ratio the total concentration of the metals was obtained by addition of the molar concentrations of the metals.

The mixtures were spotted on the filter paper strips and the chromatograms were run at constant temperature in a thermostat-controlled room at 30°; the time allowed was 90 minutes.

### *Separation of cobalt(II) and cadmium(II)*

The final concentration of each of the metal ions was 0.05 *M*; $H_2S$ water was used for developing. It was found that cobalt(II) and cadmium(II) are separated at all concentrations of tartrate up to a tartrate:metal ratio of 1:5 to 1:1. With a tartrate: metal ratio of 1 and higher, cadmium(II) does not move and only cobalt(II) diffuses upwards. For practical purposes a 1:1 concentration of tartrate is recommended, since separation is then satisfactory; the metal ions can be detected with the help of $H_2S$ water.

### *Separation of cadmium(II) and copper(II)*

The final concentration of each of the metal ions was 0.05 *M*; a freshly prepared mixture of $H_2S$ water and $K_4Fe(CN)_6$ was used for developing. In these systems precipitation is found to occur and redissolution of the precipitate is only complete when 1.7 equivalents of tartrate are added; beyond 0.9 equivalent, however, no separation is possible. It was further found that good separation of the ions from the supernatant liquid is obtained when 0.3 to 0.4 equivalent of tartrate is added, though a portion remains settled as a precipitate.

### *Separation of cadmium(II) and nickel(II)*

The final concentration of each of the metal ions was 0.05 *M*; a mixture of $H_2S$ water and dimethyl glyoxime was used for developing. In this case 60% ethanol was more efficient as it gave a better separation. For good separation of the ions 0.7 to 0.8 equivalent of the complexing agent gives the best results; no separation is possible beyond 0.9 equivalent of tartrate because of the spreading and overlapping of the zones.

### *Separation of copper(II) and nickel(II)*

The final concentration of each of the metal ions was 0.05 *M*; a mixture of $K_4Fe(CN)_6$ and dimethyl glyoxime was used for developing. At the beginning precipitation of the tartrates occurs in the systems and when about 0.9 equivalent of tartrate has been added, total redissolution of the precipitate takes place. The separation of the ions is possible when up to 0.9 equivalent of tartrate is added, but with higher concentrations no separation occurs. A good separation is obtained when 0.3 to 0.4 equivalent of the complexing agent is used.

### *Separation of cobalt(II) and copper(II)*

The final concentration of each of the metal ions was 0.05 *M*; a freshly prepared mixture of $H_2S$ water and $K_4Fe(CN)_6$ was used for developing. In these systems precipitation was found to occur and the total redissolution of the precipitate took place when 2.2 equivalents of the complexing agent was used. It was also found that the separation of copper(II) and cobalt(II) ions is possible with up to 0.9 equivalent of the complexing agent, but with higher concentrations of the complexing agent separation is not possible. A good separation is obtained with 0.3 to 0.4 equivalent of complexing agent.

### *Separation of nickel(II) and cobalt(II)*

In the case of these ions no separation is possible because both ions move simultaneously and have nearly the same $R_F$ values. Both ions can, however, be detected together by spraying with a mixture of dimethyl glyoxime and $H_2S$ water and finally exposing the chromatograms to vapours of ammonia.

### *Separation of cobalt(II), cadmium(II) and copper(II)*

The final concentration of each of the metal ions was 0.05 *M*; the indicator used was a mixture of $H_2S$ water and $K_4Fe(CN)_6$, freshly prepared. No precipitation occurs when 0.05 to 0.177 equivalent of tartrate is added, but with higher concentrations of the complexing agent precipitation occurs in the system after the solution has been allowed to stand. The separation of copper(II), cobalt(II) and cadmium(II) ions is possible when up to 0.481 equivalent of tartrate is added, but with higher concentrations of the complexing agent separation is not possible under the experimental conditions used.

### *Separation of copper(II), cadmium(II) and nickel(II)*

The final concentration of each of the metal ions was 0.05 *M*; a mixture of $H_2S$ water, $K_4Fe(CN)_6$ and dimethyl glyoxime was used as indicator. At the beginning no precipitation occurs in the system and when about 0.177 or more equivalents of the complexing agent are added precipitation occurs after the solution has been allowed to stand for some time. Of a mixture of copper(II), cadmium(II) and nickel(II) ions separation of copper(II) ion is only possible with up to 0.59 equivalent of the complexing agent; with higher concentrations separation is not possible.

### *Separation of cobalt(II), nickel(II) and copper(II)*

The final concentration of each of the metal ions was 0.05 *M*; a mixture of $K_4Fe(CN)_6$ and dimethyl glyoxime was used as indicator. No precipitation occurs with 0.059 to 0.177 equivalent of the complexing agent, but with higher concentrations precipitation occurs after the solution has been allowed to stand for a long time. Of a mixture of nickel(II), cobalt(II) and copper(II) ions, the separation of copper(II) is only possible with up to 0.59 equivalent of the complexing agent; with higher concentrations of the complexing agent no separation takes place.

### *Separation of nickel(II), cobalt(II), cadmium(II) and copper(II)*

The final concentration of each of the metal ions was 0.042 *M*; a mixture of $K_4Fe(CN)_6$, $H_2S$ water and dimethyl glyoxime was used for developing. No precipitation occurs when 0.05 to 0.15 equivalent of tartrate is added, but with higher concentrations precipitation is observed. Only the separation of copper(II) ions from cobalt(II), nickel(II) and cadmium(II) ions is possible with up to 0.65 equivalents of tartrate added. No separation occurs with higher concentrations of tartrate.

### *Separation of nickel(II), cobalt(II) and cadmium(II)*

No satisfactory separation of nickel(II), cobalt(II) and cadmium(II) ions is possible with ethanol as solvent, due to the spreading and overlapping of the zones.

## ACKNOWLEDGEMENT

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## SUMMARY

In this paper we have summarised the results obtained when an attempt was made to separate copper(II), nickel(II), cadmium(II) and cobalt(II) from mixtures by filter paper strip chromatography, using aqueous ethanol as solvent. The effect of the presence of varying concentrations of tartrate ion as complexing agent was studied. The complexing agent was added to the solution of the metals, and not to the solvent as was usually done by previous workers.

It was observed that, in general, when two metals are present, separation occurs when up to 0.9 equivalents of tartrate ions are added (ratio total metal: tartrate 1:0.9). When the amount of tartrate added is increased to 1.0 equivalent or more, no separation occurs. For good separation in the case of three metals present together, up to 0.6 equivalent of tartrate may be added. Excess of tartrate leads to overlapping of zones.

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# DIAZOREACTION FOLLOWING CHLORAMINE-T-OXIDATION AS A SENSITIVE AND SPECIFIC TEST FOR ASPARTIC ACID

HERMANN STEGEMANN AND HELEN FRANCIS GRIFFIN*
*Medizinische Forschungsanstalt der Max-Planck-Gesellschaft, Silikose-Labor, Göttingen (Germany)*

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When gelatin and bovine albumin hydrolyzates on paper chromatograms were sprayed with Chloramine-T and Ehrlich aldehyde reagents[1] as a means of identifying hydroxyproline, aspartic acid was found to give a weak pink color after several days. Even though 10 $\gamma$ aspartic acid/cm$^2$ were visible, attempts to improve this reaction failed.

In his investigation of the action of Chloramine-T upon aspartic acid, DAKIN[2] obtained dichloroacetaldehyde as the final oxidation product and noted that when the aspartic acid–Chloramine-T reaction mixture or dichloroacetaldehyde and ammonia were distilled, an unidentified compound was obtained which gave a highly colored product when coupled with diazotized sulfanilic acid[3].

We have found that, when aspartic acid on paper chromatograms is oxidized with Chloramine-T and coupled with diazotized sulfanilic acid, 1 $\gamma$/cm$^2$ is recognizable. Attempts to employ this series of reactions as a basis for a quantitative determination were unsuccessful. However, this is the only known test which permits a rapid differentiation of glutamic from aspartic acid, and with reference to the latter, the most specific and sensitive one on paper.

## MATERIALS AND METHOD

### Reagents

*Chloramine-T reagent.* N/50 Chloramine-T in 50 % methanol.

*Sodium carbonate.* 10 % solution.

*Diazo reagent.* 0.5 g semi-dry diazotized sulfanilic acid (if stored in a deepfreezer, it remains in good condition over a period of at least two years) is suspended in 5 ml cold water prior to application. The suspension should appear quite milky and is usable at least three days, if stored in a deepfreezer.

### Spraying technique

The dried chromatogram is heavily sprayed with the Chloramine-T reagent with the aid of a mechanical sprayer. The correct amount of reagent has been applied when the moist paper requires 30 to 45 min to dry in the air. No heat may be applied. The diazo reagent is applied within 45 min to 3 h after application of the Chloramine-T reagent. No moisture from the Chloramine-T reagent may remain on the paper. The sodium carbonate solution must be very sparingly applied as fine droplets under 150 to 200 mm Hg pressure, preferably at a distance of approximately 50 to 60 cm. The paper should not become more than just barely moist during application. After 5 min the diazo reagent is applied in the same manner. If the paper becomes too moist, unreacted Chloramine-T will bleach the color in 3 to 5 min, or if too much diazotized sulfanilic acid is applied, the background will become yellow. The manner of spraying the diazo reagent is especially critical. When properly applied, the diazo reagent appears as innumerable fine dots on a white background. Aspartic acid yields immediately a bluish-red or red-orange spot. The relative amounts of the two colors are principally dependent upon the time interval between application of the two reagents. More bluish-red color is obtained with shorter and more red-orange color with longer intervals. The red-orange coupled product is the more stable of the two.

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* Present address: Research Division, Columbus Psychiatric Institute, University Health Center, Columbus, Ohio, U.S.A.

## RESULTS

The comparison of the two products obtained from aspartic acid after oxidation and coupling with diazotized sulfanilic acid is given in Table I.

TABLE I

COMPARISON OF THE TWO COUPLED PRODUCTS OBTAINED FROM ASPARTIC ACID

| | *Bluish-red* | *Red-orange* |
|---|---|---|
| Sensitivity ($\gamma$Asp/cm$^2$) | 1 | 1 |
| Aqueous solution | unstable | very stable |
| Alkaline solution (1 $N$ NaOH) | unstable | very stable |
| Spectral curve (aqueous solution) | — | sharp maximum at 480 m$\mu$ |

TABLE II

REACTION OF AMINO ACIDS WITH THE CHLORAMINE-T AND THE DIAZO SPRAY REAGENTS

| *Amino acid* | *Color* | | | *Sensitivity $\gamma$/cm$^2$* |
|---|---|---|---|---|
| | *Diazo only* | *Chloramine-T only* | *Chloramine-T and diazo* | |
| Aspartic acid | none | none | bluish-red/red-orange | 1 |
| Asparagine | none | none | bluish-red | 5 |
| Glutamic acid | none | none | none | |
| Glutamine | none | none | none | |
| Cysteine | none | none | orange-red | 1 |
| Cystine | none | none | orange-red | 5 |
| Histidine | red | none | | 1 |
| | | | brown | 1 |
| Methionine | none | none | pink | 1 |
| Methionine sulfone | yellow | — | — | 1 |
| Tyrosine | red | | | 1 |
| | | yellow | | 1 |
| | | | remains yellow | |
| Tryptophan | | violet | brown | |
| Creatine | none | none | none | |
| Creatinine | yellow | none | yellow | 10 |
| Glycocyamine | none | none | none | |
| Glycocyamidine | brown | yellow | yellow | 100 |

The spectral curve was determined in a Unicam Sp. 500 in aqueous solution. A 50 γ spot of aspartic acid, treated with the Chloramine-T and diazo reagents and showing only the red-orange color, was washed with methanol to remove any excess Chloramine-T and extracted in 10 ml water. A blank was prepared in the same manner. The color intensity of the aqueous solution decayed at the constant rate of 2.5 %/h over a period of 8 h.

Table II summarizes the interfering amino acids. Other amino acids give no colored products with the Chloramine-T and diazo reagents and it should be pointed out, that neither glutamic acid nor glutamine form a colored diazo-product.

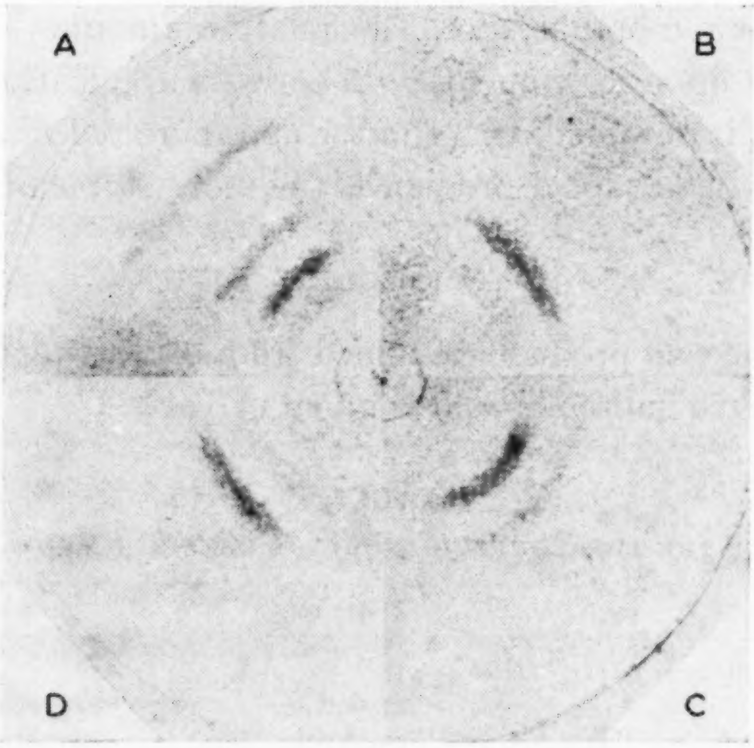

Fig. 1. Paper chromatogram on Schleicher u. Schüll 2043 b mgl. Additional explanations see Table III.

The chromatogram pictured in Fig. 1 was developed 48 h in freshly prepared *n*-butanol–glacial acetic acid–water (4:1:1)[4]. After drying, the Chloramine-T reagent was applied followed by the diazo reagent 3 h later. The identity of the bands is given

TABLE III

IDENTITY OF BANDS (See Fig. 1)

| *Substance spotted* | *Band* | *Identity* | *Relative $R_F$ Asp* | *Color* | *Absorption at 366 mμ* | *Amino acid present[a] in hydrolyzates γ* |
|---|---|---|---|---|---|---|
| A. Bovine albumin hydrolyzate 46γ | I | Unknown | 49 | Pink | — | ? |
| | II | Histidine | 69 | Brown | + | 2 |
| | III | Aspartic acid | 100 | Red-orange | + | 4 |
| | IV | Tyrosine | 172 | Yellow | + | 2 |
| B. Gelatin hydrolyzate 40γ | I | Aspartic acid | 100 | Red-orange | + | 2 |
| C. Amino acid mixture | I | Histidine | 68 | Brown | + | |
| 10γ each of Hist, Asp, Glu | II | Aspartic acid | 100 | Red-orange | + | |
| D. Aspartic acid 5γ | | | 100 | Red-orange | + | |

in Table III. Both bovine albumin* and gelatin were hydrolyzed 24 h in 20% HCl at 105°.

## DISCUSSION

Synthetic dichloroacetaldehyde gives a bluish-red color when coupled with diazotized sulfanilic acid on paper. This color is unstable in aqueous or alkaline solution. Thus the bluish-red coupled product given by aspartic acid shortly after oxidation is probably due to dichloroacetaldehyde. The time interval required for the appearance of the red-orange coupled product with the concommitant gradual disappearance of the bluish-red coupled product would indicate that the compound responsible for the bluish-red color further reacts, condensing with ammonia derived from the $-NH_2$ group in a manner similar to glyoxal[6] to yield an imidazole as suggested by DAKIN[2]. Imidazole coupled with diazotized sulfanilic acid on paper is reported to give a red-orange product[7].

## ACKNOWLEDGEMENTS

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## SUMMARY

A differentiation of aspartic acid from other amino acids, especially from glutamic acid, is possible when paper chromatograms have been sprayed with Chloramine-T and diazotized sulfanilic acid.

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* Crystalline Bovine albumin, Lot. No. 3273, Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.

# SELECTIVE GAS-LIQUID CHROMATOGRAPHIC SEPARATION OF AROMATIC COMPOUNDS WITH TETRAHALOPHTHALATE ESTERS

STANLEY H. LANGER, CHARLES ZAHN AND GUS PANTAZOPLOS
*Central Experiment Station, U.S. Bureau of Mines, Pittsburgh, Pa. (U.S.A.)*

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The problem of separating compounds of closely similar structure by gas-liquid chromatography is currently under attack from two distinct but complementary viewpoints. One is the development of more efficient columns and techniques to refine the separation process; the other is the use and development of selective liquid substrates. With the latter course, a liquid substrate is used which is sensitive to structural differences and brings about separation through these differences.

Because of the importance of aromatic materials in synthetic fuel products derived from coal and because of the difficulty in analyzing these complicated products the initial objective of this study was to develop selective liquid substrates for separating aromatic compounds. The separation of *m*- and *p*-xylene was of particular interest because until recently[1-7] these two materials, which are among the simplest aromatic isomers, had not been separated by gas chromatography. The tetrahalophthalate esters were selected as potential selective liquid substrates for aromatic compounds because they contain an aromatic ring with a large number of electronegative substituents[5]. Therefore, they would be expected to be electron acceptors in "charge transfer" interactions of the "$\pi$-$\pi$" type[8,9]. Variations in the behavior of aromatic hydrocarbons as electron donors, as well as their vapor pressures and activity coefficients[10-13] should then serve to separate these materials in the gas chromatographic column.

## EXPERIMENTAL

### *Apparatus*

A flow diagram of the gas chromatography apparatus is shown in Fig. 1. The Foxboro regulator Type 67-R222 was modified by plugging the bleed hole with solder to prevent loss of gas. The Gow-Mac thermal conductivity cell and stainless steel columns were operated in an insulated circulating air bath in which temperature was controlled to ± 0.1°. Helium was used as the carrier gas.

### *Materials*

Dimethyl tetrachlorophthalate was prepared (in low yield) by a modification of the method of KOLLONITSCH AND VITA[14] in which methyl borate-methanol azeotrope was

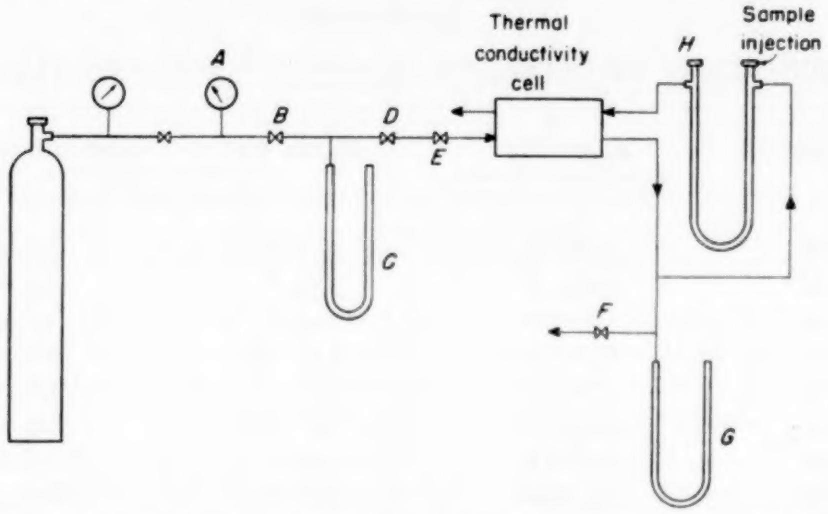

Fig. 1. Schematic diagram of apparatus for gas–liquid chromatography. *A*: reduced pressure gage. *B*: system pressure regulator valve; modified Foxboro regulator. *C*: mercury manometer. *D*: control orifice; needle valve. *E*: gas shut-off valve. *F*: pressure bleed; needle valve. *G*: mercury manometer; column pressure. *H*: chromatographic column.

initially refluxed with tetrachlorophthalic anhydride. Preparation of other tetrahalophthalates is described elsewhere[15]. Narrow mesh range firebrick was used as a solid support. The firebrick, predried in an oven at 140°, was treated with an acetone solution of the liquid phase with or without stirring under nitrogen. In later experiments, the flask used for treating the firebrick was shaken occasionally without stirring. Hydrocarbons used in the mixtures were mostly 98% pure or better as obtained from Phillips Petroleum Co., Eastman Organic Chemicals, or A.P.I. standard samples.

### *Columns*

Stainless steel U-shaped columns were connected into flow lines with Ermeto fittings (Weatherhead Co., Cleveland 8, Ohio). Long columns were constructed of 6 foot U-shaped sections connected with stainless steel capillary tubing. The 35 foot di-*n*-propyl tetrachlorophthalate column, however, was of one piece construction and carefully wound on a 2 in. mandrel. All columns were packed while being shaken by an electric vibrator. These columns are described in Table I. Conditions of operation are indicated in Table II. Data for the dimethyl tetrachlorophthalate column were obtained on an auxiliary vapor-heated apparatus.

TABLE I

COLUMNS USED IN THIS STUDY

| *Column* | *Liquid substrate* | *Packing, g* | *Percent liquid substrate (w/w)* | *Column dimensions, cm* | | *Firebrick, mesh size* |
|---|---|---|---|---|---|---|
| | | | | *length* | *diameter* | |
| A | 90% Dimethyl tetrachlorophthalate + 10% di-*n*-propyl tetrachlorophthalate | 12.1 | 25 | 120.7 | 0.45 | 35–60 |
| B | Methyl propyl tetrachlorophthalate | 65.1 | 20 | 692.0 | 0.45 | 42–48 |
| C | Di-*n*-propyl tetrachlorophthalate | 89.5 | 7 | 889.0 | 0.5 | 35–42 |
| D | Di-*n*-butyl tetrachlorophthalate | 32.7 | 10 | 304.8 | 0.5 | 42–48 |
| E | Di-*n*-propyl tetrabromophthalate | 12.3 | 10 | 181.6 | 0.45 | 42–48 |

TABLE II

CONDITIONS OF OPERATION OF CHROMATOGRAPHIC COLUMNS

| *Column* | *Temperature, °C* | $p_1$*, mm Hg* | $p_0$*, mm Hg* | *Flow rate, ml/mm** | *Pressure correction factor*** |
|---|---|---|---|---|---|
| A | 97.8 | 1167.3 | 741.8 | 35.4 | 0.765 |
| B | 110.0 | 1660.8 | 734.8 | 44.6 | 0.584 |
| | 95.0 | 1675.6 | 744.6 | 45.3 | 0.586 |
| C | 110.0 | 1725.2 | 743.2 | 88.2 | 0.572 |
| | 100.0 | 1709.0 | 740.0 | 90.0 | 0.574 |
| | 90.0 | 1674.6 | 737.6 | 84.1 | 0.582 |
| D | 110.0 | 1386.2 | 735.2 | 112.8 | 0.672 |
| | 100.0 | 1372.0 | 740.0 | 109.3 | 0.681 |
| E | 110.0 | 1020.3 | 743.3 | 48.5 | 0.836 |
| | 100.0 | 1055.7 | 742.2 | 56.8 | 0.817 |

* Flow corrected to column temperature and atmospheric pressure.

** Correction for pressure drop across column is $\frac{3}{2}\frac{(p_1/p_0)^2-1}{(p_1/p_0)^3-1}$ (ref. [17]).

### *Sample injection*

Samples were injected by means of a microsyringe of a type described earlier[16]. For sample injection, the gas flow was stopped and when column pressure fell to 100–200 mm, the bleed valve was slowly opened until the inlet of the column was depressurized. The valve was then closed, sample injected and gas flow resumed. When the marker air peak emerged, the column pressure had reached 99% of operating value.

Approximately 3 $\mu$l samples were generally used. Where a mixture contained only two or three compounds of interest the sample was diluted with more volatile materials so that the column would not be greatly overloaded for the materials of interest.

A preheater was not used for the sample injection section. For xylene separations, in which we were particularly interested, a preheater effected little or no improvement in peak resolution with our apparatus.

## DISCUSSION

Relative retention volume data for hydrocarbons on dimethyl tetrachlorophthalate, methyl *n*-propyl tetrachlorophthalate, di-*n*-propyl tetrachlorophthalate, di-*n*-butyl tetrachlorophthalate and di-*n*-propyl tetrabromophthalate are presented in Tables III and IV. Toluene was used as the standard reference material. Retention volume data for *m*- and *p*-xylene are given to 4 significant figures since special attention was given to their determination. For a particular column these values were reproducible to 1–2 parts per thousand. While absolute values did vary slightly more with different columns, relative values (for the xylenes) were close to this range of reproducibility. 10% by weight of di-*n*-propyl tetrachlorophthalate was included in the dimethyl tetrachlorophthalate, m.p. 90°, with the idea of decreasing its viscosity at the operating temperature of 97.8°. This temperature was apparently slightly high for the dimethyl

## TABLE III

RELATIVE RETENTION VOLUMES ON TETRACHLOROPHTHALATE ESTERS (TOLUENE = 1)*

| | *Methyl propyl tetrachlorophthalate* | | *Di-n-propyl tetrachlorophthalate* | | | *Di-n-butyl tetrachlorophthalate* | |
|---|---|---|---|---|---|---|---|
| | *110°* | *95°* | *110°* | *100°* | *90°* | *110°* | *100°* |
| Benzene | 0.459 | 0.43 | 0.455 | 0.439 | 0.421 | 0.451 | 0.435 |
| Toluene | (1.000)[a] | (1.000)[b] | (1.000)[c] | (1.000)[d] | (1.000)[e] | (1.000)[f] | (1.000)[g] |
| *m*-Xylene | 2.038 | 2.158 | 2.045 | 2.125 | 2.203 | 2.071 | 2.152 |
| *p*-Xylene | 2.122 | 2.255 | 2.125 | 2.217 | 2.302 | 2.144 | 2.231 |
| *o*-Xylene | 2.71 | — | 2.70 | 2.81 | — | 2.71 | 2.84 |
| Ethylbenzene | 1.70 | 1.76 | 1.74 | 1.79 | 1.82 | 1.77 | 1.81 |
| Styrene | — | — | 2.82 | 2.93 | — | 2.80 | — |
| Phenylacetylene | — | — | 2.68 | 2.81 | 2.20 | 2.60 | 2.72 |
| Propylbenzene | — | — | 3.12 | 3.28 | — | 3.19 | 3.34 |
| Cumene | — | — | 2.45 | 2.56 | — | 2.49 | 2.60 |
| 1-Methyl-2-ethylbenzene | — | — | 4.31 | 4.63 | — | 4.40 | 4.72 |
| 1-Methyl-3-ethylbenzene | — | — | 3.37 | 3.57 | — | 3.46 | 3.67 |
| 1-Methyl-4-ethylbenzene | — | — | 3.53 | — | — | 3.61 | 3.83 |
| Mesitylene | — | — | 3.85 | 4.14 | — | 3.98 | 4.28 |
| 1,2,4-Trimethylbenzene | — | — | 5.32 | — | — | 5.41 | 5.89 |
| Hemimellitene | — | — | 6.88 | 7.49 | — | 6.96 | — |
| Butylbenzene | — | — | 6.29 | 6.89 | — | 6.45 | 7.03 |
| Heptane | 0.20 | — | 0.25 | 0.24 | 0.22 | 0.26 | 0.24 |
| Octane | 0.42 | — | — | — | 0.49 | 0.54 | 0.53 |
| Nonane | — | — | — | — | — | 1.10 | 1.12 |
| Decane | 1.71 | — | — | — | — | 2.24 | 2.38 |
| Heptene | — | — | 0.27 | 0.26 | — | 0.28 | 0.27 |
| Octene | 0.47 | — | 0.56 | 0.55 | 0.59 | 0.58 | 0.57 |
| Nonene | — | — | 1.14 | 1.16 | — | 1.19 | 1.22 |
| Decene | 1.93 | — | 2.33 | 2.43 | — | 2.43 | 2.58 |
| 2-Methylhexane | — | — | 0.20 | 0.19 | — | 0.21 | 0.19 |
| 2-Methylheptane | — | — | 0.40 | 0.39 | — | 0.42 | 0.41 |
| 2-Methyloctane | — | — | 0.83 | 0.83 | — | 0.87 | 0.88 |
| 2-Methylnonane | — | — | 1.70 | 1.74 | — | 1.79 | 1.87 |
| Cyclohexane | — | — | — | — | — | — | 0.21 |
| Methylcyclohexane | 0.32 | — | 0.37 | 0.35 | 0.33 | 0.39 | 0.37 |
| Dimethylcyclohexane | 0.71 | — | — | — | — | — | 0.85 |
| Butanol | ~ 0.47 | — | ~ 0.31 | — | — | — | — |
| 2-Butanone | ~ 0.27 | — | — | — | — | — | — |

* Retention volume of air taken as zero. Corrected retention volumes in ml/g of liquid phase, for columns of Table I at column temperature: a = 142, b = 215, c = 162, d = 221, e = 290, f = 163, g = 217.

ester since some difficulty was encountered with condensation of the solid ester when the capillary tubing connecting the column to the cell (heated separately) was not heated.

The data of Tables III and IV make it possible to characterize the tetrahalophthalates qualitatively as liquid phases for gas chromatography.

### *Selectivity*

The tetrahalophthalates are selective for aromatic materials in that aliphatic compounds of the same boiling range are eluted from the column first. (They are not as selective for aromatics as the dipropionitriles from which decane emerges long before

TABLE IV

RELATIVE RETENTION VOLUMES ON TETRAHALOPHTHALATE ESTERS (TOLUENE = 1)*

| | *Di-n-propyl tetrabromophthalate* | | *Dimethyl tetrachlorophthalate-90% + di-n-propyl tetrachlorophthalate-10%* |
|---|---|---|---|
| | *110°* | *100°* | *97.8°* |
| Benzene | 0.470 | 0.451 | 0.45 |
| Toluene | (1.000)[a] | (1.000)[b] | (1.000)[c] |
| *m*-Xylene | 1.967 | 2.023 | 2.076 |
| *p*-Xylene | 2.030 | 2.094 | 2.167 |
| *o*-Xylene | 2.66 | 2.80 | 2.85 |
| Ethylbenzene | 1.72 | 1.76 | 1.72 |
| Styrene | 2.86 | 3.01 | — |
| Phenylacetylene | 2.82 | 2.94 | — |
| Propylbenzene | 3.03 | 3.17 | — |
| Cumene | 2.38 | 2.46 | — |
| 1-Methyl-2-ethylbenzene | 4.21 | 4.50 | — |
| 1-Methyl-3-ethylbenzene | 3.22 | 3.40 | — |
| 1-Methyl-4-ethylbenzene | 3.36 | 3.55 | — |
| Mesitylene | 3.51 | 3.73 | — |
| 1,2,4-Trimethylbenzene | 4.95 | 5.39 | — |
| Hemimellitene | 7.26 | 7.87 | — |
| Butylbenzene | 5.97 | 6.45 | — |
| Heptane | 0.21 | 0.20 | 0.16 |
| Octane | 0.42 | 0.43 | 0.33 |
| Nonane | 0.87 | 0.88 | — |
| Decane | 1.76 | 1.83 | 1.41 |
| Heptene | 0.24 | 0.23 | — |
| Octene | 0.48 | 0.48 | — |
| Nonene | 1.00 | 1.01 | — |
| Decene | 2.00 | 2.11 | — |
| 2-Methylhexane | 0.17 | 0.16 | — |
| 2-Methylheptane | 0.34 | 0.33 | — |
| 2-Methyloctane | 0.68 | 0.69 | — |
| 2-Methylnonane | 1.40 | 1.46 | — |
| Methylcyclohexane | 0.35 | 0.33 | — |
| Butanol | — | 1.30 | — |
| 1-Propanol | — | — | 0.22 |

* Retention volume of air taken as zero. Corrected retention volume in ml/g of liquid phase at column temperature: a = 101, b = 134, c = 159.

benzene[18]. However, the use of dipropionitriles is limited by their volatility.) The selectivity of the tetrahalophthalates appears to be controllable to some extent since it is governed by the proportion of alkoxyl group comprising the ester. Whereas octane is eluted before benzene from dimethyl tetrachlorophthalate and methyl propyl tetrachlorophthalate, it is eluted after benzene from di-*n*-butyl and di-*n*-propyl tetrachlorophthalate. Increasing the atomic weight of the halogen has an effect similar to decreasing the size of the alkoxyl group and octane emerges before benzene from di-*n*-propyl tetrabromophthalate.

Naphthenes and olefins are retained longer than paraffins of comparable boiling point while alcohols and ketones emerge earlier and exhibit marked peak distortion due to tailing. The retention volumes of these oxygenates is greater when the alkoxyl groups are smaller; this may be due to increased accessibility and concentration of the polar ester groups.

For the saturated hydrocarbons and olefins on the substrates reported here, the same general observations apply as reported by others. Retention volumes are affected in a regular manner by addition of methylene groups to the solute molecule and the logarithm of retention volume or relative retention volume varies linearly with carbon number.

*Effect of temperature on selectivity*

While the relative retention volumes of heptane with respect to benzene are lowered with a decrease in temperature, the geometrical factor for addition of a methylene group increases (as temperature decreases) and the relative retention volumes for the higher paraffins tend to be greater compared with aromatic materials. Therefore, type separations of aliphatic and aromatic compounds are not improved by decreasing operating temperature.

*Separations of aromatic hydrocarbons*

Typical separations of aromatic hydrocarbons on di-*n*-propyl tetrabromophthalate at 100° and di-*n*-propyl tetrachlorophthalate at 100° and 110° are shown in Figs. 2 and 3. These may be compared with separations of similar mixtures on naphthalene tetracarboxylic acid ester[19] and Apiezon L[20]. Neither substrate effected separation of

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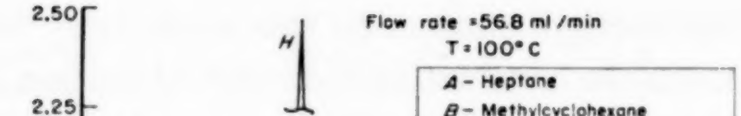

Fig. 2. Separation of hydrocarbon mixture on propyl tetrabromophthalate 10%. Column = 0.45 × 181.6 cm.

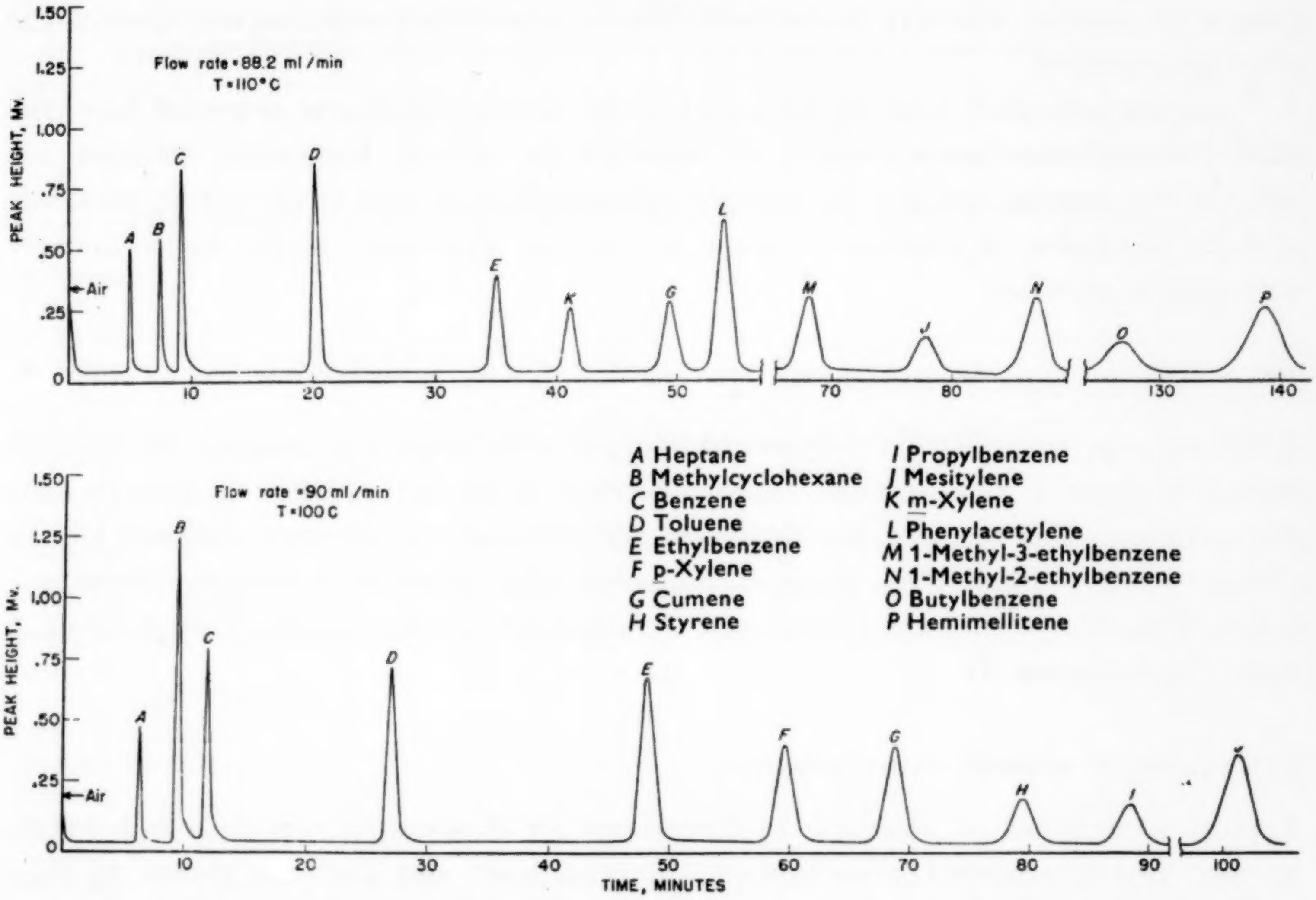

Fig. 3. Gas chromatographic separation of hydrocarbons on propyl tetrachlorophthalate. Column = 0.5 × 889 cm. 89.5 g packing containing 7% (w/w) liquid on 35–42 mesh firebrick.

mesitylene and 1-methyl-2-ethylbenzene. In this work, they were separated by both propyl tetrahalophthalates. In contrast to the order of elution shown in Figs. 2 and 3, retention data reported for Apiezon L[20] indicate mesitylene emerges after 1-methyl-2-ethylbenzene.

The data of Tables III and IV show that, although the relative retention volumes of the aromatic hydrocarbons on various tetrahalophthalates do not vary greatly, definite though sometimes minor trends exist. Thus, as the amount of alkyl group in the alkylbenzenes becomes greater, retention volumes relative to toluene increase with increasing percentage of alkoxyl group in the tetrahalophthalate ester. This is not true for phenylacetylene, styrene, and benzene, where presumably activity coefficients increase (and elution times decrease) with increasing aliphatic content of the tetrahalophthalate ester. At the same time, the difference between styrene and phenylacetylene is emphasized and the separation factor is improved by going from either propyl tetrabromophthalate or propyl tetrachlorophthalate to butyl tetrachlorophthalate. Thus, a particular separation of two compounds of different type can often be achieved without upsetting the general order of elution by choosing a partitioning liquid which is a higher (or lower) member of a homologous series.

*Separation of* m- *and* p-*isomers*. It is readily shown[10–13, 21] that the relative volatility, $\alpha_{1,2}$, or separation factor for two substances from the partitioning liquid may be expressed as

$$\alpha_{1,2} = \gamma_1^0 p_1^0 / \gamma_2^0 p_2^0 \qquad (1)$$

where $\gamma^0$ is the activity coefficient at infinite dilution and $p^0$ is the vapor pressure of the pure solute.

For initial discussion purposes, the measured separation factor or relative volatility of two aromatic isomers is treated in terms of

$$a_{1,2} = g_1 p_1{}^0 / g_2 p_2{}^0 \tag{2}$$

where $g_1$ and $g_2$ are "apparent activity coefficients" which may or may not include correction terms for complex formation, depending on definition of the latter. Since neither $\gamma_1$ nor $g_1$ have been measured $g_1$ and $g_2$ are treated relative to each other.

Vapor pressures of a number of aromatic compounds at several temperatures are presented in Table V and the separation factors for *m*- and *p*-xylene on several liquid phases are given in Table VI. On the basis of vapor pressure alone it would be expected that *p*-xylene would emerge from a gas chromatographic partitioning liquid before *m*-xylene. Indeed, JAMES AND MARTIN[22] found this to be true for a number of substrates including paraffin wax, a polyglycol and benzyldiphenyl. ZLATKIS and coworkers[7] have recently confirmed this order of elution from benzyldiphenyl in a report on the xylene separation.

TABLE V

VAPOR PRESSURES OF AROMATIC COMPOUNDS*

| | *Vapor pressure in mm at* | | |
|---|---|---|---|
| | *110°* | *100°* | *90°* |
| Benzene | 1756.9 | 1350.7 | 1021.7 |
| Toluene | 746.6 | 556.3 | 406.7 |
| Ethylbenzene | 355.3 | 257.0 | 181.9 |
| *o*-Xylene | 277.4 | 198.5 | 139.0 |
| *m*-Xylene | 324.5 | 233.6 | 164.6 |
| *p*-Xylene | 333.2 | 240.4 | 169.8 |
| *n*-Propylbenzene | 177.5 | 124.7 | 85.62 |
| Cumene | 218.6 | 155.0 | 107.4 |
| 1-Methyl-2-ethylbenzene | 146.0 | 101.6 | 68.99 |
| 1-Methyl-3-ethylbenzene | 161.4 | 114.6 | 78.18 |
| 1-Methyl-4-ethylbenzene | 162.1 | 113.4 | 77.49 |
| 1,2,3-Trimethylbenzene | 103.2 | 70.81 | 47.36 |
| 1,2,4-Trimethylbenzene | 127.4 | 88.14 | 59.48 |
| 1,3,5-Trimethylbenzene | 145.5 | 101.0 | 68.38 |
| *n*-Butylbenzene | 82.32 | 55.89 | 36.95 |
| Styrene | 268.0 | 191.1 | 133.4 |

* Calculated from Antoine's equation[23].

The emergence of *p*-xylene after *m*-xylene from the tetrahalophthalates as illustrated in Figs. 4 and 5 is additional evidence[4, 5] that "charge transfer" forces are operative between the solute (electron donor) and the tetrahalophthalate (electron acceptor). *p*-Xylene has the lower base ionization potential and would be expected generally to interact more strongly[8, 24, 25] (or form the more stable complex) with the tetrahalophthalate so that it would be held up in the chromatographic column.

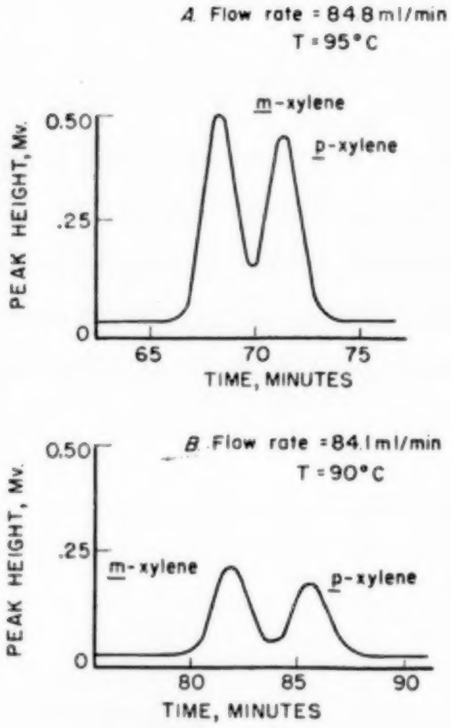

Fig. 4. Separation of *m*- and *p*-xylene on propyl tetrachlorophthalate. Column = 0.5 × 889 cm. 89.5 g packing containing 7% (w/w) liquid on 35–42 mesh firebrick.

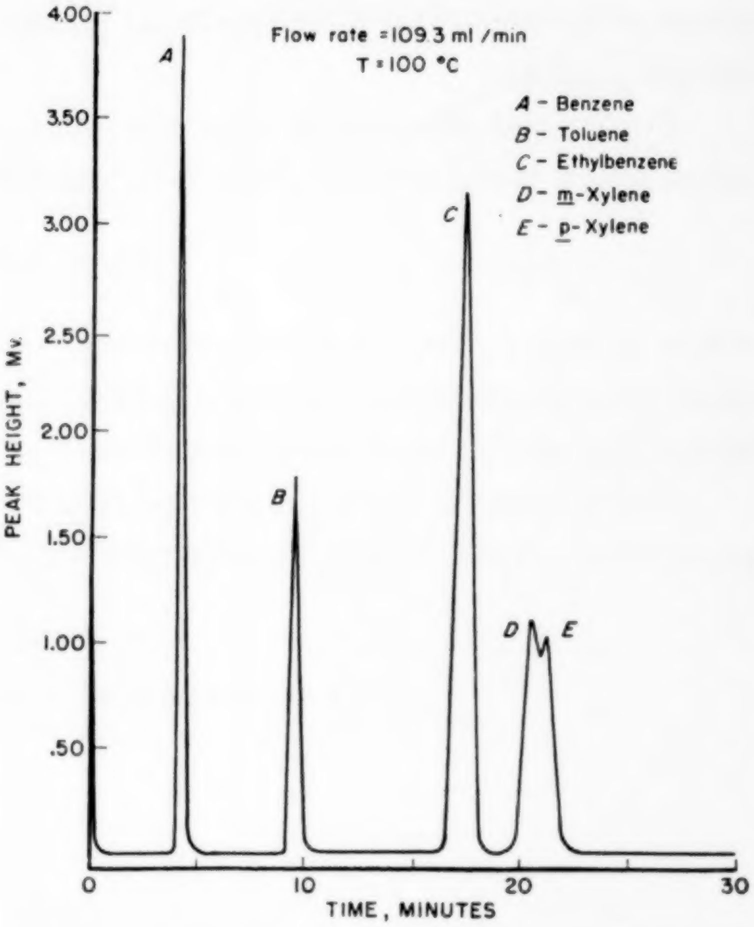

Fig. 5. Separation of aromatic hydrocarbon mixture on butyl tetrachlorophthalate. Column = 0.5 × 304.8 cm. 32.7 g packing with 10% (w/w) liquid on 42–48 mesh firebrick.

Since vapor pressure relationships favor emergence of the *p*-xylene before *m*-xylene, favorable separation factors based on differences in basicity of these aromatic hydrocarbons are difficult to attain, *i.e.* the effect of vapor pressures and base strengths are opposed.

From the vapor pressure data, separation factors and equation (2), $g_1/g_2$ is 1.067 and 1.078, for *m*- and *p*-xylene on propyl tetrachlorophthalate at 110° and 90°

TABLE VI

SEPARATION FACTORS FOR *m*- AND *p*-XYLENE ON TETRAHALOPHTHALATE ESTERS

| *Liquid substrate* | *Temperature °C* | *Separation factor p-/m-* |
|---|---|---|
| Dimethyl tetrachlorophthalate (90%) + di-*n*-propyl tetrachlorophthalate (10%) | 97.8 | 1.044 |
| Methyl propyl tetrachlorophthalate | 110.0 | 1.041 |
| | 95.00 | 1.045 |
| Di-*n*-propyl tetrachlorophthalate | 110.0 | 1.039* |
| | 100.0 | 1.043 |
| | 90.0 | 1.045 |
| Di-*n*-butyl tetrachlorophthalate | 110.0 | 1.035 |
| | 100.0 | 1.037 |
| Di-*n*-propyl tetrabromophthalate | 110.0 | 1.032 |
| | 100.0 | 1.035 |

* This value is a revision of the earlier value of 1.042 reported in ref.[5]. This may be due to experimental error and/or the fact that the earlier value was determined on a column with 20% liquid phase.

respectively. The separation factors of Table VI indicate that separation of *m*- and *p*-xylene is favored by lower temperatures despite the fact that the ratio of vapor pressure becomes more unfavorable for separation. The increase in separation factor with decreasing temperature, therefore, is a net effect also which is dominated by the $g_1/g_2$ increase.

The separation of 0.7 μl and 0.4 μl samples of *m*- and *p*-xylene mixtures on di-*n*-propyl tetrachlorophthalate is shown in Fig. 4, A and B respectively. The peaks are separated by 4 and 4.4 standard deviations[26] on a 35 foot column operated at an efficiency (based on toluene) of 7,500 and 7,000 plates respectively. Separation of a 3.5 μl aromatic mixture on a 12 foot butyl tetrachlorophthalate column is shown in Fig. 5. Although the column is somewhat overloaded a partial separation of *m*- and *p*-xylene is achieved in less than 25 min at an operating efficiency of 3,500 plates.

For the tetrachlorophthalates of Table VI, the favorable xylene separation factor apparently diminishes with increasing aliphatic content of the molecule. This is as expected since *p*-xylene emerges before *m*-xylene from an aliphatic liquid phase[22]. As the nature and composition of the ester molecule changes, the nature of the interaction with solute molecules changes accordingly. For the bromophthalate, the separation factor for *m*- and *p*-xylene was probably smaller than for the chlorophthalates because of the lower electronegativity of bromine. Therefore, "charge-transfer" interaction is less intense and its effect is diminished. A similar diminution in complex stability has been noted in a comparison of aromatic complexes of tetrachlorophthalic and tetrabromophthalic anhydrides[27].

The separation factor, *ca.* 1.048, for 1-methyl-3-ethylbenzene and 1-methyl-4-ethylbenzene on propyl tetrachlorophthalate is more than adequate for separating these previously unresolved isomers[19, 20]. While their vapor pressure ratios (see Table V) are slightly more favorable for separation by "charge transfer" interaction, the $g_1/g_2$ ratio (observed) is slightly less favorable. However, the net effect permits sufficient resolution for quantitative analysis of mixtures of these materials. We have obtained a separation of 4.5 standard deviations on the propyl tetrachlorophthalate column.

*Quantitative analysis.* To demonstrate the usefulness of the tetrahalophthalate liquid substrates, a commercial "xylene" mixture was analyzed on the 27 foot column containing 20 % methyl propyl tetrachlorophthalate as reported previously[5]. Analysis was based on peak heights and the xylene mixture was diluted with a low boiling hydrocarbon so that the 5,900 plate column was not overloaded (maximum 0.8 μl of either xylene isomer). Despite the fact that the xylene peaks were separated by 3.3 standard deviations, which should be adequate for analysis by peak heights[26], successful analysis was not possible without calibration with several mixtures because of the slight asymmetry of the *m*-xylene peak.

A calibration curve for 3.6 μl samples containing 25 % toluene and various amounts of the xylene isomers is shown in Fig. 6. While the *m*-xylene peak heights were independent of the *p*-xylene concentration, the reverse was not true. Points A and B represent observed *p*-xylene peak heights in the presence of *m*-xylene. From such observations it was possible to correct the *p*-xylene peak height for the slight contribution of *m*-xylene. When 20 % of *m*-xylene was present in the 3.6 μl sample, the correction to the *p*-xylene peak height was about 3 % of the observed *m*-xylene peak height. After correction, analysis of the commercial mixture could be checked by means of a synthetic blend within the limits of reproducibility of our recorder, 0.003 mV.

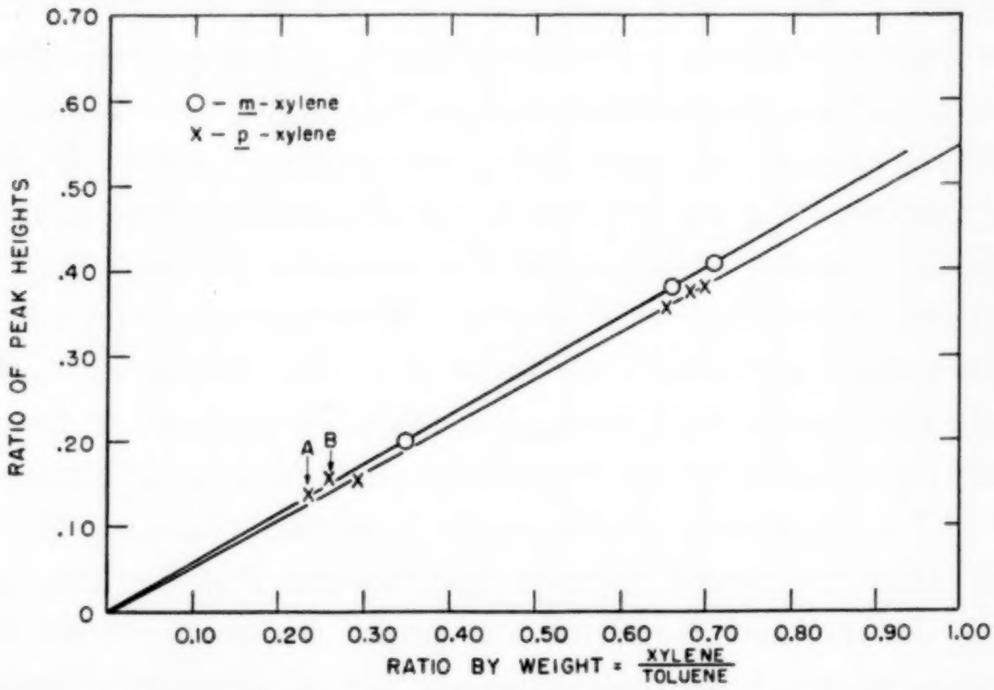

Fig. 6. Calibration curve for analysis of xylene mixtures using toluene as an internal standard.

### *Complexing*

While solid molecular complexes of the tetrahalophthalate esters have been isolated[15], the question of whether and to what extent complexes *per se* account for the abnormal retention volumes of aromatic materials on the tetrahalophthalates is difficult to answer and is greatly dependent on definition. A reasonable amount of evidence exists[5, 15] that "charge transfer" forces are involved[1, 22], but these may be interpreted as contributing to van de Waals forces as initially suggested by MULLIKEN[28]. The difficulties encountered in attempting to interpret spectral data, for example, in terms of "charge transfer" complexes have recently been discussed by ORGEL AND MULLIKEN[29]. In any event, sufficient data were not obtained in this study to evaluate equilibrium constants for "complex" formation. However, these data are adequate for obtaining a qualitative picture of the tendency of some of the aromatic hydrocarbons to interact or "complex" with the tetrahalophthalate liquid phases. For convenience, this interaction will be discussed in terms of complex formation in this section.

The corrected retention volume $V_R^0$, taken from the time of *emergence of the air peak* is[11, 12, 17, 30]

$$V_R^0 = k'V_s \tag{3}$$

where $V_s$ is the volume occupied by the liquid phase in the gas chromatographic column and $k'$ is a pseudo solute partition coefficient defined (here) as the ratio of solute per unit volume of liquid phase, including such material as may be complexed, to solute per unit volume of the gas phase *for very dilute solutions.*

Then, defining the activity coefficient, $\gamma$, for the solute[11, 30, 31]

$$yP = \gamma x p^0 \tag{4}$$

where $P$ is pressure of the gas phase (ideal), $y$ is mol fraction of solute in the gas, and $x$ is mol fraction of *uncomplexed* solute in the liquid phase. For very dilute solutions where the concentration of liquid phase is essentially constant

$$k' = x'M_s/yM_m \tag{5}$$

where $M_s$ is moles of stationary phase per unit volume, $M_m$ is moles of mobile gas per unit volume, and $x'$ is total solute in solution complexed and uncomplexed.

If

$$x = (1 - c)\, x' \tag{6}$$

where $c$ is the fraction of material complexed, then from eqn. (5) following others[11, 30]

$$k' = M_s RT/(1 - c)\, \gamma p^0 \tag{7}$$

and the separation factor for two solutes on a column becomes

$$\alpha_{1,2} = (1 - c_1)\, \gamma_1 p_1{}^0/(1 - c_2)\, \gamma_2 p_2{}^0 \tag{8}$$

and the $g$ of eqn. (2) is

$$g = (1 - c)\, \gamma \tag{9}$$

The difficulty is in resolving $\gamma$ and $1 - c$, one of which must be measured independently. However, eqn. (7) permits conclusions about relative complexing tendency or the strength of "charge transfer" interaction by considering isomeric aromatic compounds. For example, for the $C_9$ saturated alkylbenzenes it would seem reasonable to assume, as a first approximation, that bulk interaction (activity coefficient) with the tetrahalophthalate liquid substrate would be the same except for variation in interaction between the aromatic ring and the tetrahalophthalate nucleus. The more closely any two alkylbenzenes resemble each other, the more nearly true is this assumption.

Table VII shows an arrangement of $C_9$ alkylbenzenes in the order of values of $(1 - c)$ for di-*n*-propyl tetrachlorophthalate at 110°. The $(1 - c)$ values were calculated from eqn. (7), the vapor pressure values given in Table V, and the relative

TABLE VII

RELATIVE $(1 - c)$ FACTORS FOR $C_9$ ALKYLBENZENES (CUMENE = 1)

| | *Relative fraction of material not complexed* $(1-c)$ |
|---|---|
| Cumene | (1.00) |
| 1-Methyl-3-ethylbenzene | 0.99 |
| Propylbenzene | 0.97 |
| Mesitylene | 0.96 |
| 1-Methyl-4-ethylbenzene | 0.94 |
| 1-Methyl-2-ethylbenzene | 0.85 |
| 1,2,4-Trimethylbenzene | 0.79 |
| Hemimellitene | 0.76 |

retention values of Table IV. The $\gamma$ values are assumed to be equal for the saturated $C_9$ alkylbenzenes and the value of $(1 - c)$ for cumene was arbitrarily taken as 1. The arrangement then, is in the order of increasing tendency to "complex" or interact depending on definition. This order is as expected if the interaction is assumed to be between the parallel molecules. Thus, propylbenzene has a greater tendency to complex than cumene.

For the methylethylbenzenes the order of complexing (or basicity) is *o*- > *p*- > *m*-. For the three trimethylbenzenes the complexing order can be interpreted in terms of a steric factor. Molecular models show that in the di-*n*-propyl tetrachlorophthalate molecule, crowding is such that the ester groups tend to orient out of the plane of the ring with the carbonyl oxygen and alkoxyl group on one side or the other. In a parallel complex there could be some steric interference from methyl groups on the aromatic donor which correspond to either of the *ortho* ester groups. The order of basicity hemimellitene (1,2,3-trimethylbenzene) > 1,2,4-trimethylbenzene > mesitylene gives some credence to this idea. For mesitylene, there are no two adjacent positions on the aromatic ring which are free of methyl groups; thus, the close approach and advantageous orientation of the aromatic ring relative to the tetrachlorophthalate ester which is possible for the other trimethylbenzenes appears to be excluded.

## ACKNOWLEDGEMENT

We wish to express our thanks to PAUL GOLDEN and his group for construction of our apparatus and to PETER PANTAGES and MARGHARET VIAL for their assistance with this study. We are also grateful to Dr. J. HOWARD PURNELL for many helpful discussions during the course of this work.

## SUMMARY

Tetrahalophthalate esters were found to be selective liquid substrates for separating aromatic hydrocarbons by gas chromatography The tetrachlorophthalates are unusually specific in separating *meta*- and *para*-isomers, including the xylenes; this makes possible the gas chromatographic analysis of mixtures of these isomers. "Charge transfer" interaction between the volatile aromatic compounds (donors) and the tetrahalophthalates (acceptors) is apparently responsible for selectivity. Variations in the elution order of alkylaromatics may be interpreted qualitatively in terms of structure or molecular composition. Gas chromatography, in turn, provides a measure of the complexing or degree of interaction of aromatic compounds with the tetrahalophthalate esters as well as information on salient stereochemical features that effect interaction.

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# ANALYSE DES AMINES BIOLOGIQUES PAR LES TECHNIQUES DE CHROMATOÏONOPHORÈSE, ÉLECTROPHORÈSE ET CHROMATOGRAPHIE SUR PAPIER

J. BLASS ET A. SARRAFF

avec la collaboration technique de

MARIE BLANCHE NICOLAS

*Institut Pasteur, Annexe de Garches, Service de Chimie Bactérienne, Garches (France)*

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L'étude présente a été entreprise dans le but de déterminer les amines biologiques produites dans un milieu de culture par action bactérienne.

Dans trois publications antérieures[1-3] l'un de nous a décrit l'application des techniques de chromatoïonophorèse et d'électrophorèse à l'analyse de quelques amines biologiques fixes, présentes dans un mélange à côté d'amino-acides.

Nous avons étendu ces techniques à une série d'amines biologiques fixes ou volatiles pouvant se trouver dans les bouillons de culture.

La technique de chromatoïonophorèse proposée permet l'identification des amines fixes dans le bouillon de culture, après élimination des bases aminées volatiles, sans nécessiter leur extraction préalable. Elle permet également une meilleure résolution d'un mélange d'amines extraites du bouillon, que ne le font la technique simple d'électrophorèse sur papier[4, 5] ou de chromatographie sur papier[6-9] proposées pour leur analyse.

A côté d'amines biologiques fixes nous avons étudié une série d'amines volatiles. Divers auteurs ont appliqué les procédés de chromatographie et d'électrophorèse sur papier à leur identification[4,7-13].

Nous décrivons les procédés qui nous ont permis d'obtenir les meilleurs résultats.

## MATÉRIEL ET MÉTHODES

### *A. Amines biologiques fixes*

Les amines suivantes ont été étudiées (sous forme de chlorhydrates ou sulfates en solution 0.01 *M*): cadavérine, putrescine, agmatine, histamine, tyramine* et tryptamine.

L'étude des amines biologiques fixes est effectuée dans le bouillon débarrassé de corps bactériens, après élimination des bases aminées volatiles (voir chapitre Amines volatiles).

---

* Amines pour lesquelles GALE[14] a isolé les décarboxylases responsables de leur formation à partir des acides $\alpha$-aminés correspondants.

Le bouillon privé des bases volatiles est analysé avant et après hydrolyse. Celle-ci est effectuée en présence de HCl 6 *N*, pendant 24 heures à 110°, en ampoule scellée sous vide. L'hydrolysat est évaporé dans un dessiccateur sous vide, en présence de $H_2SO_4$ et de KOH en pastilles. On chasse l'acide chlorhydrique en ajoutant un peu d'eau et en évaporant à nouveau, ainsi à trois reprises. On reprend l'hydrolysat par un volume d'eau correspondant au volume initial du bouillon. Le bouillon est soumis avant et après hydrolyse à l'analyse préalable par électrophorèse sur papier, puis par chromatoïonophorèse. On dépose sur le papier des volumes variables du bouillon, pouvant contenir jusqu'à 500 $\mu$g d'azote total (10 à 50 $mm^3$ dans nos essais).

### *Électrophorèse sur papier*

Nous avons étudié la séparation des bases aminées dans les tampons suivants:

| | |
|---|---|
| tampon de pH = 8.6: | véronal sodique 10.3 g, véronal acide 1.84 g, eau pour compléter à 1000. |
| tampon de pH = 4.0 à base de phtalate: | phtalate acide de potassium 0.1 *N* 100 ml, NaOH 0.1 *N* 0.8 ml, eau pour compléter à 200. |
| tampon de pH = 4.0 à base d'acide sulfosalicylique[5]: | acide sulfosalicylique 0.05 *M*. On amène à pH = 4.0 par addition de soude*. |
| tampon de pH = 10.0: | ammoniaque 0.065 *N* (7 ml de $NH_3$ concentré dans 1 l). |
| tampon de pH = 11.7**: | ammoniaque *N*. |
| tampon de pH = 2.4**: | acide acétique *N*. |

On utilise pour l'électrophorèse des bandes de papier Whatman No. 3. On dépose les solutions à analyser, ainsi que les solutions témoins, sur une ligne distante de 7 cm de la ligne médiane, vers le bord anodique, ces lignes étant perpendiculaires à la marche du courant. On utilise à côté des témoins latéraux, des témoins internes.

L'électrophorèse est pratiquée dans l'appareil de MACHEBOEUF, REBEYROTTE ET DUBERT***, sous 400V, pendant un temps variable, suivant la nature du tampon (2 à 5 h).

Le papier est ensuite séché à l'étuve à 100°. On révèle les amines en trempant les bandes dans une solution de ninhydrine à 0.1 % dans l'acétone[15] et en les séchant pendant quelques minutes à l'étuve à 100°. Dans le cas du tampon pH = 8.6, on ajoute à la solution acétonique de ninhydrine 1 % d'acide acétique (v/v) (solution fraîchement préparée). On peut aussi utiliser une solution de ninhydrine à 0.1 % dans du butanol saturé d'eau, additionnée de 1 % d'acide acétique (solution stable) et opérer par pulvérisation. On obtient des colorations violettes, bleues ou gris-violettes suivant la nature de l'amine.

### *Chromatoïonophorèse*

Nous utilisons pour la chromatoïonophorèse une feuille de papier Whatman No. 3[16] de 57 cm × 46.5 cm. On effectue dans la première dimension une chromatographie descendante, et dans la deuxième dimension une électrophorèse sur papier, le sens du courant étant perpendiculaire au sens d'écoulement du solvant.

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* Ce tampon ne peut pas être utilisé pour le bouillon non hydrolysé.

** Ces deux tampons ne nous ont pas donné de résultats satisfaisants.

*** Lérès, 9 cité Canrobert, Paris XV°.

On dépose la solution à analyser en O (voir Fig. 1) à 7 cm de la ligne médiane M N, vers le bord qui sera plongé dans le compartiment anodique, et à 11 cm du bord A B. On traces les lignes E F et G H suivant la largeur de l'appareil à électrophorèse qui doit recevoir ces bandes (soit 32 cm largeur maximum K L dans nos essais).

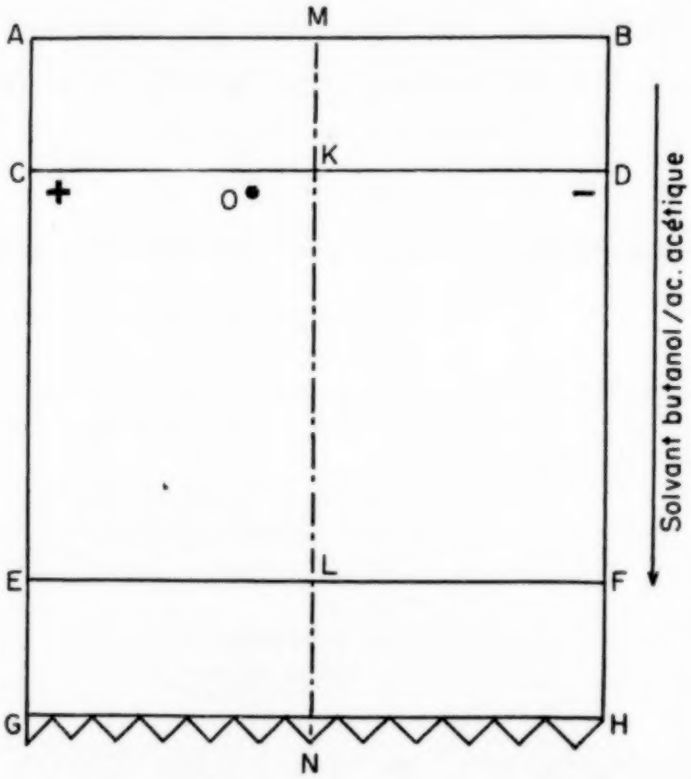

Fig. 1.

Le solvant utilisé pour la chromatographie est le mélange butanol–acide acétique–eau, formule de Woiwod[17]*.

On laisse migrer le solvant 24 à 32 h. En laissant plus de 24 h, le solvant commence à s'écouler du papier, mais la base la plus rapide, tryptamine ($R_F$ = 0.66) ne quitte généralement pas le papier, après 32 h de chromatographie.

Les bandes CDEF et EFGH découpées sont placées dans l'appareil à électrophorèse, en utilisant un des tampons cités plus haut, le tampon pH = 8.6 de préférence. Le papier est mouillé avec la solution tampon en laissant une marge de 4 cm de chaque côté de la position présumée des amino-acides et des bases aminées et on laisse le papier s'imbiber complètement par capillarité. On poursuit l'ionophorèse comme il a été décrit plus haut.

A côté du révélateur général, constitué par la solution de ninhydrine, nous avons utilisé pour quelques amines (histamine, tyramine, tryptamine et agmatine) des révélateurs spécifiques.

| | |
|---|---|
| Histamine et tyramine: | Réactif de Pauly: acide sulfanilique diazoté, formule de Dent[6]. |
| Tryptamine: | Réactif d'Ehrlich, formule de Smith[15]. |
| Agmatine: | Réactif de Sakaguchi, formule de Roche *et al.*[8] ou formule de Jepson et Smith[18]. |

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* Butanol 125 ml, acide acétique 30 ml, eau 125 ml.

### *B. Bases aminées volatiles*

Les amines suivantes ont été étudiées (sous forme de chlorhydrates):

*Amines primaires*: en solution 0.004 $M$ (concentration la plus convenable): méthylamine, éthylamine, propylamine, butylamine, isobutylamine, isoamylamine, phényléthylamine et allylamine.

*Amines secondaires*: diméthylamine en solution 0.01 $M$, diéthylamine en solution 0.1 $M$ et diisoamylamine en solution 0.04 $M$.

*Amines tertiaires*: triméthylamine en solution 0.2 $M$ et triéthylamine en solution 0.02 $M$.

On déposait sur le papier 5 à 10 mm³ de ces solutions.

Nous utilisons pour la chromatographie du papier Whatman No. 1 ou No. 4, tamponné avec une solution d'acétate de soude 0.8% et comme solvant le mélange butanol–acide acétique–eau (125:30:125).

On révèle les amines primaires et secondaires avec une solution de ninhydrine à 0.1 %, additionnée de 1 % d'acide acétique (voir plus haut). On sèche à l'étuve à 100° au moins 20 min car les amines secondaires ne se révèlent qu'après un chauffage prolongé. On obtient des colorations violettes, bleues et rouges suivant la nature de l'amine.

Pour la révélation spécifique des amines non saturées on peut utiliser le révélateur proposé par DIHLMANN[10,11]: (solution fraîche à 1% de $\beta$-naphtoquinone sulfonate de soude dans une solution de carbonate de soude à 5%), on obtient une coloration bleue pour les amines saturées et une coloration jaune-verte pour les amines non saturées.

On révèle les amines tertiaires par immersion avec le réactif de CHARGAFF[19,20] (1. acide phosphomolybdique à 2%, lavage avec du butanol pendant 5 min, puis avec eau courante pendant 5 min, 2. solution de $SnCl_2$ à 0.4% dans HCl 3 $N$). Les amines tertiaires donnent des taches bleu-foncé sur un fond bleu-clair.

Pour identifier les bases aminées volatiles présentes dans le bouillon de culture, on distille le bouillon débarrassé de corps microbiens, après alcalinisation à pH = 8.0[21]. Le distillat est recueilli dans un récipient refroidi à 0°, puis neutralisé avec acide sulfurique sans excès, et convenablement concentré par distillation sous vide. On ramène le volume du bouillon privé de bases volatiles à sa valeur initiale par addition d'eau distillée.

## RÉSULTATS

### *A. Bases aminées fixes*

#### (*a*) *Chromatographie sur papier*

Le Tableau I indique les $R_F$* des bases aminées étudiées, chromatographiées dans le solvant butanol–acide acétique, sur papier Whatman No. 3, les limites de sensibilité de leur réaction avec la solution de ninhydrine ainsi qu'avec les révélateurs spécifiques.

---

* Remarquons que les $R_F$ dans le solvant butanol–acide acétique sont légèrement variables d'une chromatographie à l'autre.

TABLEAU I

| *Base aminée* | $R_F$ | *Révélateur à la ninhydrine, limite de sensibilité en μg* | *Révélateurs spécifiques, limite de sensibilité en μg* |
|---|---|---|---|
| Agmatine | 0.18 | 0.2 | 0.5 |
| Cadavérine | 0.15 | 0.4 | |
| Histamine | 0.15 | 1.0 | 0.25 |
| Putrescine | 0.12 | 0.4 | |
| Tryptamine | 0.66 | 1.5 | 0.5 |
| Tyramine | 0.61 | 1.5 | 2.0 |
| Ethanolamine | 0.32 | 0.1 | |
| Glucosamine | 0.20 | 0.25 | 0.5* |

* On utilise pour la révélation de la glucosamine les réactifs de ELSON ET MORGAN, formule de PARTRIDGE ET WESTALL[22].

A titre de comparaison nous avons porté sur le même tableau les caractéristiques des deux amines suivantes: éthanolamine et glucosamine.

Voici les $R_F$ de quelques amino-acides déposés sur le même chromatogramme que les amines du Tableau I: lysine 0.13, arginine 0.17, alanine 0.32, tyrosine 0.43, phénylalanine 0.58 et leucines 0.61.

On voit que la chromatographie simple ne permet pas d'identifier les bases aminées en présence d'amino-acides. Elle ne permet pas non plus, en absence d'amino-acides, la résolution complète d'un mélange des bases aminées.

### (*b*) *Électrophorèse sur papier*

Le Tableau II indique les chemins parcourus en cm par les bases aminées, à partir de la ligne de départ, au cours de l'électrophorèse dans divers tampons, sur papier Whatman No. 3.

A titre de comparaison nous avons fait figurer dans ce tableau les deux amines

TABLEAU II

| *Base aminée* | *Tampon pH = 8.6 durée 2½ h* | *Tampon pH = 4.0 "acide sulfosalicylique" durée 2½ h* | *Tampon pH = 4.0 "phtalate" durée 2 h* | *Tampon pH = 10.0 durée 3 h* |
|---|---|---|---|---|
| Agmatine | 22.5 | 15.4 | 16.0 | 15.2 |
| Cadavérine | 23.5 | 17.2 | 17.6 | 15.0 |
| Histamine | 16.0 | 16.2 | 17.1 | 9.0 |
| Putrescine | 24.3 | 18.7 | 18.9 | 12.6 |
| Tryptamine | 12.6 | 11.4 | 8.9 | 8.8 |
| Tyramine | 14.4 | 12.3 | 10.2 | 6.0 |
| Ethanolamine | 20.8 | 17.9 | 16.5 | 8.5 |
| Glucosamine | 9.0 | 12.7 | 8.5 | 3.9 |
| Arginine | 12.4 | 11.4 | 8.3 | 5.4 |
| Lysine | 13.4 | 12.4 | 10.2 | 4.7 |

suivantes: glucosamine et éthanolamine, ainsi que les acides aminées basiques: lysine et arginine.

On voit d'après ce tableau que l'électrophorèse simple peut servir de recherche préalable pour l'analyse des amines en présence d'acides aminés car dans la plupart des tampons essayés, quelques bases aminées se déplacent plus loin que les amino-acides basiques (lysine et arginine).

L'électrophorèse seule ne permet pas cependant la résolution complète d'un mélange des bases aminées.

Notons que les chiffres indiqués pour le cheminement des bases aminées ont un caractère purement indicatif, ils ne sont valables que pour les solutions pures et les concentrations données. En présence d'un grand excès d'autres substances, les vitesses de cheminement des bases sont considérablement ralenties. La comparaison avec des témoins latéraux n'est donc pas suffisante.

Lorsqu'on cherche à identifier les bases contenues dans un volume de bouillon pouvant contenir jusqu'à 500 $\mu$g d'azote total, on dépose sur le papier, côte à côte, le bouillon seul et le bouillon additionné de divers témoins, car *seul l'emploi des témoins internes se confondant avec les taches correspondant au mélange analysé est valable pour leur identification.*

### (*c*) *Chromatoïonophorèse sur papier*

Nous donnons dans la Fig. 2 le schéma de la séparation d'un mélange d'acides aminés et de bases aminées obtenue par la technique décrite plus haut, en utilisant pour l'électrophorèse le tampon pH = 8.6.

On voit sur le schéma que toutes les bases aminées analysées sont séparées entre elles et suffisamment séparées des amino-acides basiques et neutres.

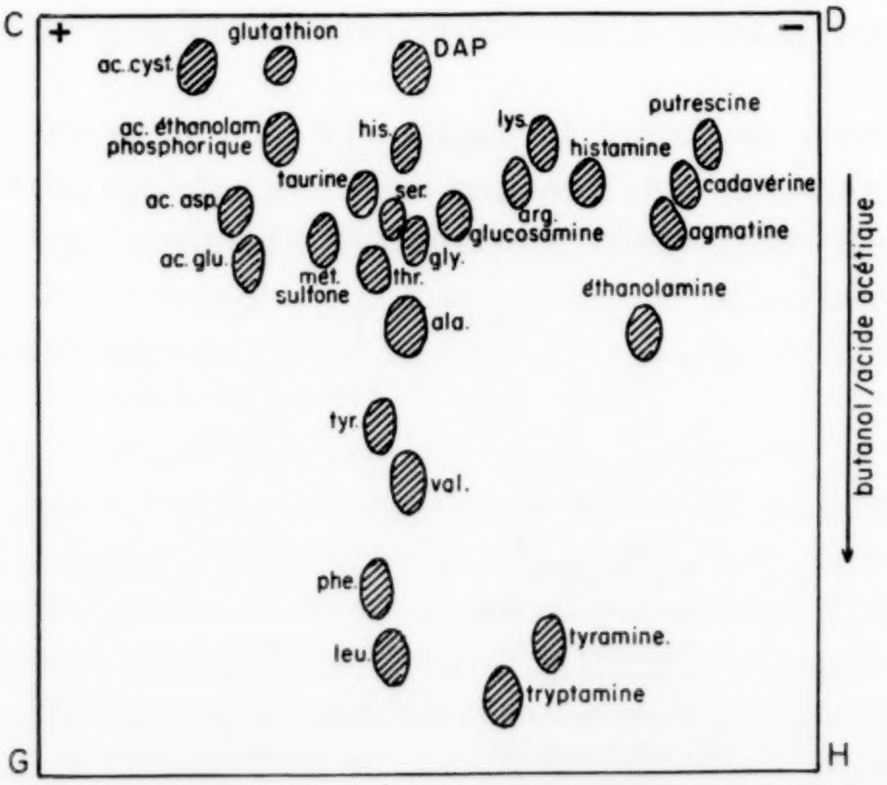
C
+
glutathion
D
−
ac. cyst.
DAP
putrescine
ac. éthanolam phosphorique
his.
lys.
histamine
taurine
ser.
cadavérine
ac. asp.
arg.
glucosamine
agmatine
ac. glu.
mét. sulfone
thr.
gly.
éthanolamine
ala.
butanol/acide acétique
tyr.
val.
phe.
tyramine.
leu.
tryptamine
G
H

Fig. 2.

## *B. Amines volatiles*

### 1. *Amines primaires et secondaires*

(*a*) *Chromatographie sur papier* (*Whatman No. 1*) *tamponné*. On trouve dans le Tableau III les $R_F$ des diverses amines étudiées, chromatographiées dans le solvant butanol–acide acétique. Les concentrations utilisées étaient indiquées dans le chapitre Méthodes.

Nous indiquons également la limite de sensibilité de la réaction avec la ninhydrine, ainsi que la teinte de la coloration obtenue.

TABLEAU III

| *Amine* | $R_F$ | *Révélateur à la ninhydrine, limite de sensibilité en μg* | *Coloration avec la ninhydrine* |
|---|---|---|---|
| Méthylamine | 0.40 | 0.15 | rouge-violette |
| Éthylamine | 0.55 | 0.10 | violette |
| Propylamine | 0.64 | 0.15 | violette |
| Isobutylamine | 0.76 | 0.2 | violette |
| Butylamine | 0.79 | 0.35 | violette |
| Isoamylamine | 0.86 | 0.10 | violette |
| Phényléthylamine | 0.84 | 0.5 | bleue |
| Allylamine | 0.61 | 0.6 | bleue |
| Diméthylamine | 0.45 | 0.2 | rosée |
| Diéthylamine | 0.66 | 18 | rosée |
| Diisoamylamine | 0.95 | 30 | rosée |

On voit sur le Tableau III que trois groupes d'amines saturées suivantes ont des $R_F$ rapprochés et ne se séparent pas bien lorsqu'elles se trouvent mélangées: méthylamine et diméthylamine, butylamine et isobutylamine, propylamine et diéthylamine.

Nous avons réussi à séparer ces amines entre elles en utilisant une bande de papier de 80 cm (papier au mètre), tamponné avec acétate de soude 0.8% et en laissant la chromatographie se poursuivre pendant 40 h (système descendant, à écoulement libre).

(*b*) *Électrophorèse sur papier*. Le Tableau IV indique les chemins parcourus en cm par les amines étudiées dans le tampon à pH = 4.0 (à base de phtalate) sur papier Whatman No. 1. L'électrophorèse a été poursuivie pendant 1½ h.

TABLEAU IV

| *Amine* | *Chemin parcouru en cm* |
|---|---|
| Méthylamine | 19.5 |
| Éthylamine | 14.5 |
| Propylamine | 12.4 |
| Butylamine | 11.2 |
| Isobutylamine | 11.2 |
| Isoamylamine | 10.2 |
| Allylamine | 13.0 |
| Diméthylamine | 15.5 |
| Diéthylamine | 11.6 |
| Phényléthylamine | 8.5 |

On voit que l'électrophorèse réalise une assez bonne séparation entre ces amines (sauf pour butylamine et isobutylamine).

## 2. *Amines tertiaires*

(*a*) *Chromatographie sur papier.* Les $R_F$ de deux amines tertiaires étudiées, chromatographiées dans les mêmes conditions que les amines primaires et secondaires, étaient: 0.29 pour triméthylamine et 0.70 pour triéthylamine.

(*b*) *Électrophorèse sur papier.* Le Tableau V indique les chemins parcourus en cm par ces deux amines dans le tampon de pH = 4.0, à base de phtalate, sur papier Whatman No. 1, ainsi que la limite de sensibilité avec le révélateur de CHARGAFF.

L'électrophorèse a été poursuivie pendant 1 ½ h.

A titre de comparaison nous indiquons sur le même tableau les résultats obtenus dans les mêmes conditions pour les bases quaternaires: choline et neurine pour lesquelles on utilise le même révélateur.

TABLEAU V

| *Amine* | *Chemin parcouru en cm* | *Révélateur de* CHARGAFF, *limite de sensibilité en µg* |
|---|---|---|
| Triméthylamine | 16.0 | 60.0 |
| Triéthylamine | 11.7 | 9.0 |
| Neurine | 14.6 | 2.5 |
| Choline | 13.4 | 10.0 |

Notons que la sensibilité de la révélation est plus grande pour les taches obtenues sur électrophorégrammes que pour celles correspondantes sur chromatogrammes car les premières sont moins étalées par rapport aux secondes. Pour l'identification des bases aminées tertiaires l'utilisation de l'électrophorèse est donc préférable à la chromatographie.

## DISCUSSION

BLOCK *et al.*[23] ont décrit une méthode d'extraction des amines d'un mélange complexe à l'aide d'éther, dans un appareil à extraction continue liquide–liquide. Cette méthode comporte plusieurs opérations, elle est de longue durée et se prête mal à des essais en série.

La méthode de chromatoïonophorèse proposée, appliquée à l'analyse des amines biologiques fixes dans le bouillon de culture, débarrassé de corps bactériens et privé d'amines volatiles, permet l'identification des amines fixes en présence d'autres substances ninhydrine-positives, sans nécessiter leur extraction préalable. Si l'on utilise cependant pour l'analyse le bouillon non hydrolysé, on risque de confondre des peptides basiques éventuellement présents avec des amines. Une comparaison entre les résultats obtenus pour le bouillon, avant et après hydrolyse acide, permet d'éliminer les taches correspondant aux peptides basiques.

Nous avons controlé que seule la tryptamine est détruite au cours de l'hydrolyse acide, toutes les autres amines étudiées sont quantitativement retrouvées.

Cette méthode facilite les analyses en série, grâce à sa simplicité et à sa rapidité. Elle permet de déceler jusqu'à 0.2 à 2 $\mu$g d'amine individuelle (suivant la nature de l'amine), dans un volume de bouillon pouvant contenir 500 $\mu$g d'azote au maximum.

Divers autres auteurs ont proposé des techniques combinées de chromatographie et d'électrophorèse sur papier, appliquées à l'analyse des amino-acides, en utilisant l'électrophorèse en première direction (avec un tampon volatil) et la chromatographie en seconde direction[24–26].

Dans notre cas particulier nous avons préféré le procédé inverse que nous avons déjà proposé en 1954[2] pour les raisons suivantes:

Le chemin parcouru par les bases, au cours d'une électrophorèse, est considérablement ralenti lorsqu'on dépose sur papier une grande quantité de substances accessoires. En soumettant à l'électrophorèse un mélange déjà séparé, par chromatographie sur papier, en plusieurs fractions, on évite en grande partie cet inconvénient.

Parmi les divers procédés proposés pour la chromatographie sur papier des amines volatiles, c'est celui de STEINER et coll.[12], utilisant des papiers tamponnés à l'acétate de soude d'après MUNIER[27] qui nous a donné les meilleurs résultats.

Si l'on utilise des papiers non tamponnés, on obtient pour les amines des taches avec front irrégulier quelque soit la formule du solvant utilisé ce qui rend la méthode moins sensible et contribue à une moins bonne séparation.

Seul l'emploi des papiers tamponnés à l'acétate de soude avec le solvant butanol–acide acétique nous a permis d'obtenir des taches bien rondes et d'augmenter ainsi la sensibilité de la révélation avec la ninhydrine. (Nous avons légèrement modifié le procédé de STEINER en utilisant, pour tamponner le papier, une solution d'acétate de soude à 0.8 % au lieu de 0.2 %.)

La sensibilité de la révélation des amines primaires et secondaires a été encore accrue par l'addition de 1 % d'acide acétique à la solution de ninhydrine à 0.1 ou 0.2 % (dans l'acétone ou butanol). Les amines secondaires, en concentration convenable, sont bien révelées, en utilisant cette formule de révélateur, à condition de chauffer au moins 20 min à 100°. Dans les mêmes conditions le révélateur proposé par DAVIES *et al.*[13] (ninhydrine à 0.2 % dans isopropanol, additionné de 20 % de pyridine) ne donne aucune coloration avec les amines secondaires. Le révélateur de CHARGAFF proposé par STEINER et coll.[12] se prête mieux à la révélation des amines tertiaires que les vapeurs d'iode proposées par DIHLMANN[11] d'après BRANTE[28].

## RÉSUMÉ

Nous décrivons une méthode de chromatoïonophorèse permettant la recherche des amines biologiques fixes, dans un bouillon débarrassé de corps microbiens, après séparation des amines volatiles et ne nécessitant pas leur extraction préalable.

Des quantités de l'ordre de quelques $\mu$g d'amines présentes dans un volume de bouillon pouvant contenir jusqu'à 500 $\mu$g d'azote, peuvent ainsi être decelées.

Nous décrivons un procédé de chromatographie sur papier des amines volatiles primaires, secondaires et tertiaires, utilisant des papiers tamponnés, ainsi qu'un procédé d'électrophorèse sur papier, et nous indiquons la limite de sensibilité de leur révélation.

## SUMMARY

A chromato-ionophoretic method is described by means of which non-volatile biological amines produced in broth culture can be studied after removal of bacterial substances and separation of volatile amines. Preliminary extraction of the non-volatile amines is not necessary.

Quantities of amines of the order of a few μg present in a volume of broth containing up to 500 μg of nitrogen, can be detected by this method.

A paper-chromatographic method using buffered papers and a paper-electrophoretic method are described for the detection of volatile primary, secondary and tertiary amines. The detection limits of these amines are given.

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# ANION EXCHANGE STUDIES

## XXVI. A COLUMN METHOD FOR MEASUREMENT OF ION EXCHANGE EQUILIBRIA AT HIGH TEMPERATURE. TEMPERATURE COEFFICIENT OF THE $Br^-$–$Cl^-$ EXCHANGE REACTION*

KURT A. KRAUS, RICHARD J. RARIDON** AND DAVID L. HOLCOMB***
*Chemistry Division, Oak Ridge National Laboratory, Oak Ridge, Tenn. (U.S.A.)*

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Although measurement of ion exchange equilibria as a function of temperature is of considerable importance for an understanding of ion exchange, relatively little work has so far been reported. Further, in spite of currently great interest in ion exchange processes above the boiling point of water, quantitative information in this region seems to be completely lacking. This paucity of information presumably is partly due to the fact that simple equipment for ion exchange measurements at elevated temperatures has not been available. It now appears that an adaptation of the preloaded column technique, used in this laboratory for some time for measurement at room temperature of very large distribution coefficients[3] overcomes this difficulty. It is the purpose of the present paper to describe the technique and newly developed equipment and to illustrate its use with a typical anion exchange reaction, the exchange of tracer bromide ions for chloride ions using a strongly basic anion exchange resin.

In the preloaded column technique a sample of fine-mesh resin, previously uniformly loaded with respect to the ion whose adsorption is to be studied, is placed into a column. Eluent passing through the column, rapidly comes to equilibrium in the upper layers of the bed. With high distribution coefficients $D$ only relatively small amounts of the adsorbed material need to leave the resin in each column volume of effluent. Hence, at sufficiently slow flow rates, the composition of the bulk of the bed remains essentially unchanged and distribution coefficients may readily be computed from analysis of the effluent solution and the known initial composition of the resin. Equilibration rates within the bed may be made extremely fast. At high values of $D$ the rate is controlled principally by diffusion through the interstitial space of the bed.

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[*] This document is based on work performed for the U. S. Atomic Energy Commission at the Oak Ridge National Laboratory, Oak Ridge, Tenn., operated by Union Carbide Corporation. Previous papers: XXIV and XXV, see ref.[1,2].

[**] Based on work submitted by R. J. RARIDON to Vanderbilt University in partial fulfilment for the requirement for a Ph. D. degree. Work carried out at Oak Ridge National Laboratory under sponsorship of the Oak Ridge Institute of Nuclear Studies. Present address, Memphis State University, Department of Physics, Memphis, Tenn.

[***] Engineering and Mechanical Division, Oak Ridge National Laboratory.

The time needed for equilibration varies with the square of the linear diffusion length which may be made extremely small by choice of small mesh particles.

This column method also has the advantage that it readily lends itself to temperature control and even to measurements above the boiling point of water. By utilizing capillary columns, relatively simple pressurized equipment can be developed.

For operation below the boiling point of the solvent the equipment can be extremely simple. As illustrated in Fig. 1, it could consist of a glass column inside a jacket through which thermostatic liquid is pumped. In the arrangement, Fig. 1, the column can easily be replaced; it is supported within the jacket by a small rubber ring which also serves as a seal for a small amount of water placed in the annulus between the column and the jacket for better heat conduction.

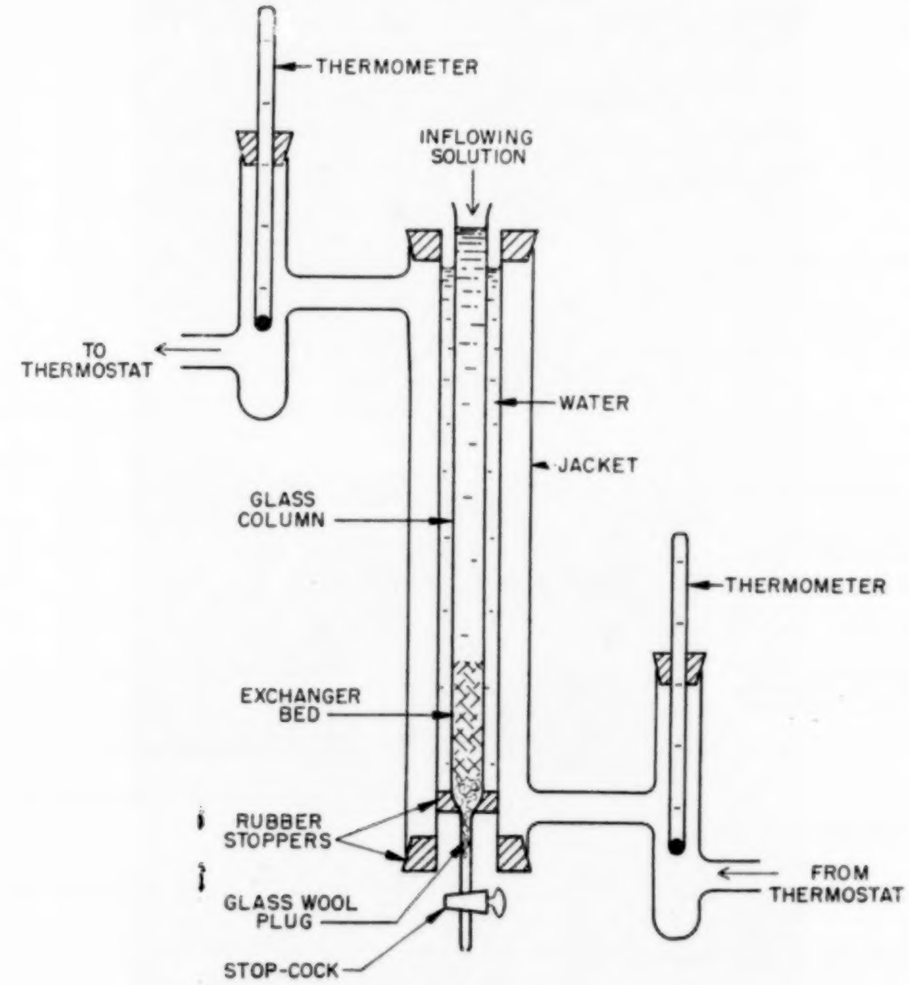

Fig. 1. Jacketed ion exchange column.

In the development of equipment suitable for measurements above the boiling point of the solvent the principle of a jacketed column has been retained. The two major design problems involved development of a simple letdown valve which could be used with ease even at slow rates for sampling the effluent under pressure and development of high pressure fittings for connecting the column to a solution reservoir in which the eluent is stored. To simplify design, all valves and fittings are near room temperature and only the bed at high temperature. Thick-walled glass capillaries were chosen for the columns to permit direct observation of the bed even under relatively high pressure. Based on these principles equipment was first built with stainless steel valves and a stainless steel solution reservoir. Another apparatus was then constructed where only glass or plastic tubings were exposed to the solutions.

It was used in the bromide–chloride exchange experiments which will be described below as well as in other experiments with solutions corrosive to stainless steel. We shall describe the principal features of the "stainless steel column" and then describe the alterations used in the all glass-plastic design.

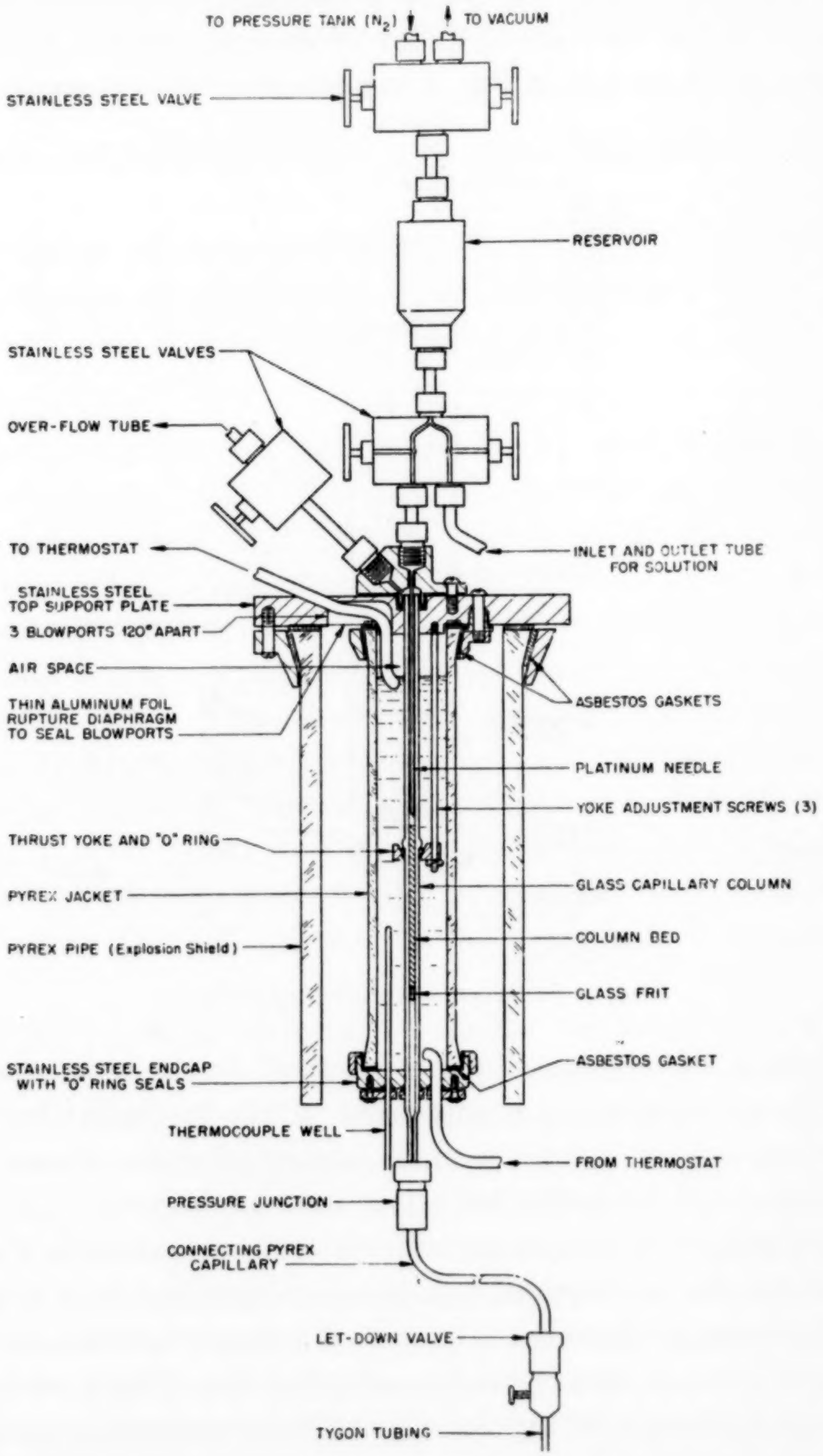
TO PRESSURE TANK ($N_2$)
TO VACUUM
STAINLESS STEEL VALVE
RESERVOIR
STAINLESS STEEL VALVES
OVER-FLOW TUBE
TO THERMOSTAT
INLET AND OUTLET TUBE FOR SOLUTION
STAINLESS STEEL TOP SUPPORT PLATE
3 BLOWPORTS 120° APART
AIR SPACE
ASBESTOS GASKETS
THIN ALUMINUM FOIL RUPTURE DIAPHRAGM TO SEAL BLOWPORTS
PLATINUM NEEDLE
YOKE ADJUSTMENT SCREWS (3)
THRUST YOKE AND "O" RING
PYREX JACKET
GLASS CAPILLARY COLUMN
PYREX PIPE (Explosion Shield)
COLUMN BED
GLASS FRIT
ASBESTOS GASKET
STAINLESS STEEL ENDCAP WITH "O" RING SEALS
THERMOCOUPLE WELL
FROM THERMOSTAT
PRESSURE JUNCTION
CONNECTING PYREX CAPILLARY
LET-DOWN VALVE
TYGON TUBING

Fig. 2. High temperature pressurized ion exchange column.

### 1. *High temperature pressurized ion exchange column*

The "stainless steel" equipment is illustrated in the diagram, Fig. 2, and the photograph, Fig. 3. This equipment has been used up to 200° and under 250 lb./in.$^2$ pressure.

The column proper is a thick-walled glass capillary with 2.5 mm I.D. and 9.0 mm O.D. The resin bed is supported by a glass frit sealed inside the capillary. A pressure seal is formed at the top of the column with a compressed "O" ring which fits snugly around the column. A yoke with an "O" ring cushion, attached to the top plate, prevents the column from being thrust out under pressure.

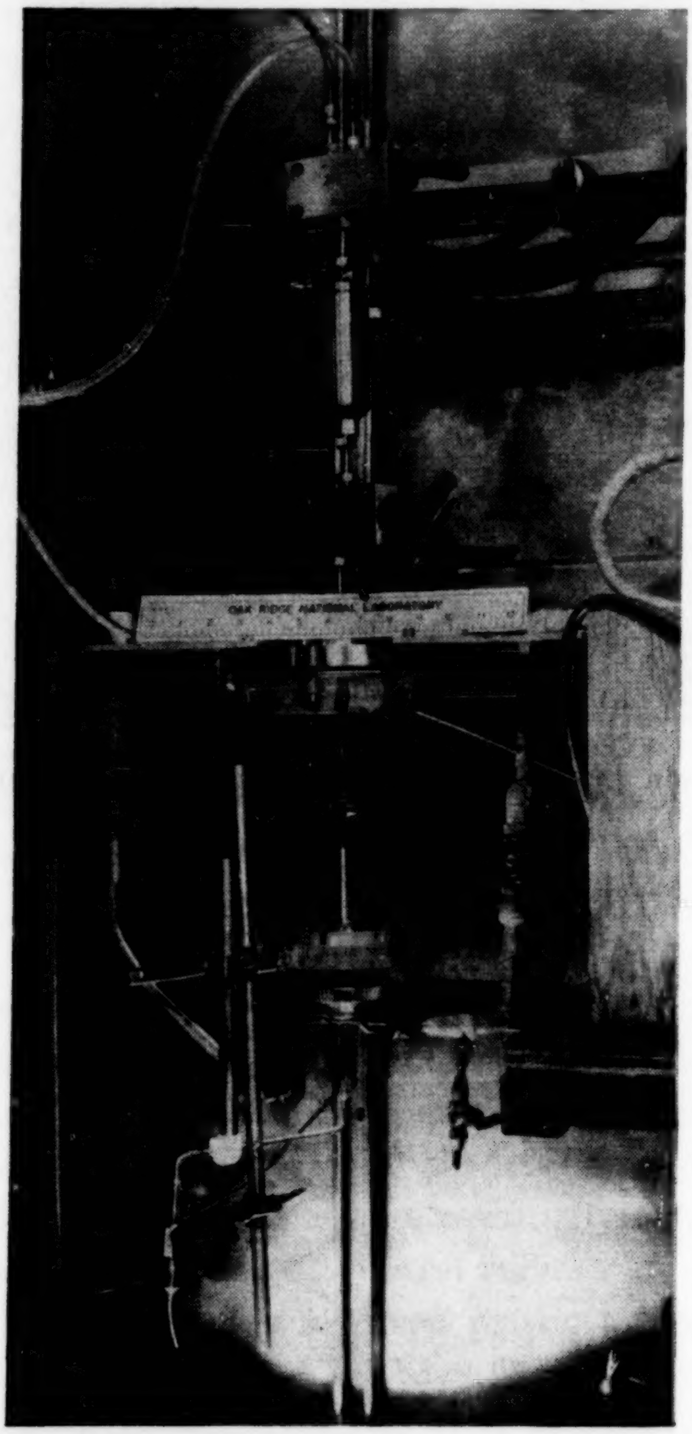

Fig. 3. High temperature pressurized ion exchange column.

The eluting solution is placed in a stainless steel reservoir which is kept near room temperature. The solution in the reservoir can be changed while the column is kept under pressure. The reservoir is isolated from the column with a stainless steel needle valve and solution is discharged under pressure through a side valve; the reservoir is filled through the same valve by applying a mild vacuum. The "old" solution can also be removed from the top portion of the column by opening a flush valve that allows the solution to flow back up the column after it has been forced down through a platinum needle. By this method trapped air may also be removed.

The bottom of the column, narrowed to a 6 mm O.D. capillary, is coupled by a pressure junction to a glass capillary (6 mm O.D., 0.5 mm I.D.) that leads to a letdown (or sampling) valve. Details of the junction and valve are shown in the photograph, Fig. 4, and diagram, Fig. 5. When the connector nuts on the pressure junction are tightened, the "O" ring between the tapered glass ends is compressed, forming a seal. The ring is held in place by a metal sleeve. The other "O" rings serve to cushion the

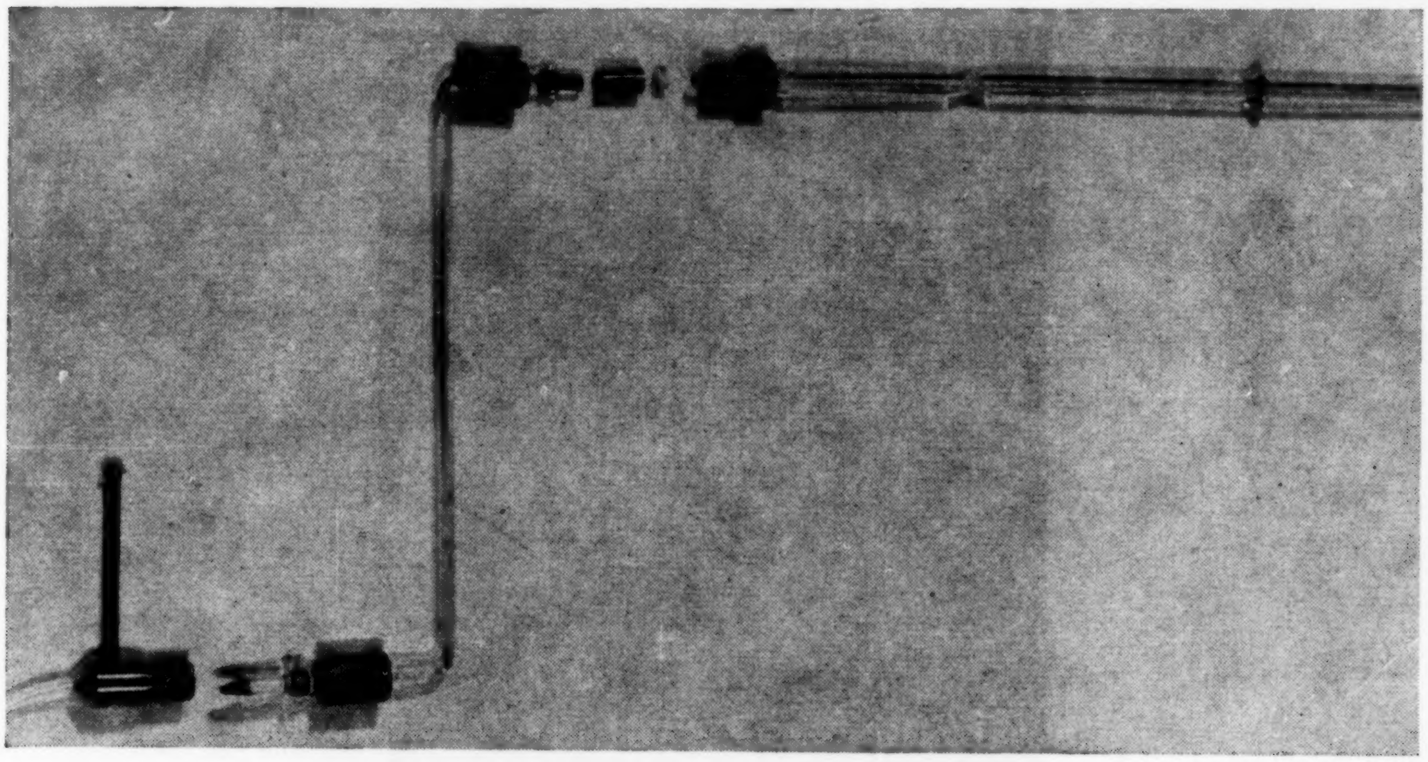

Fig. 4. Column, pressure junction, exit capillary, and letdown valve.

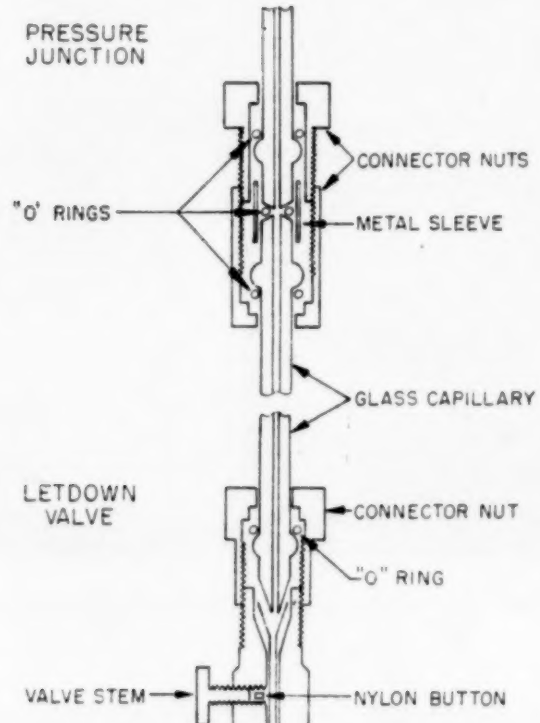
PRESSURE JUNCTION
CONNECTOR NUTS
"O" RINGS
METAL SLEEVE
GLASS CAPILLARY
LETDOWN VALVE
CONNECTOR NUT
"O" RING
VALVE STEM
NYLON BUTTON
TYGON TUBING

Fig. 5. Details of pressure junction and letdown valve.

nuts against the glass. The connector nut on the letdown valve forces the tapered end of the capillary into the flared end of a plastic (*e.g.*, "Tygon") tubing forming a seal. Deformation of the plastic tubing is prevented by a metal sleeve (aluminium) which also serves to hold a screw, the valve stem. When the valve is tightened a nylon button compresses the plastic tubing, thus controlling effluent flow.

The temperature of the column is maintained by circulating silicone oil through a Pyrex pipe surrounding the column. Oil flow is upward through the jacket. It is withdrawn through a tube projecting *ca.* 1 in. below the top plate so that an air gap separates this plate from the hot liquid, leaving the plate at relatively low temperature. The air lock above the oil in the jacket is closed by an aluminum gasket which covers three large ports leading from the jacket to a large diameter Pyrex pipe. If the column should break at high pressure, this gasket would rupture and release the pressure. The oil would then be confined and blow downward in the outer pipe. To further protect the operator, lucite panels were installed in front of the apparatus, which was built into a floor hood. The letdown valve is adjusted through a hole in one panel. The exit Tygon tubing also passes through a small hole so that samples can be collected in front of the shield.

A thermocouple well is inserted into the jacket through the bottom plate and sealed with a compressed "O" ring. Temperature is recorded continuously on a "Brown" recorder which also serves as a regulator by means of a built-in mercury switch in line with one of three heaters immersed in the oil bath. Lag in the temperature response from the bath to the column was sufficiently small to prevent fluctuations of more than ± 0.1°.

Presence of stainless steel limits use of the column to nitrate and other non-corrosive solutions. For work with chloride solutions and other solutions corrosive to stainless steel an adaptation of the equipment, employing only glass, plastic and silicone rubber in contact with solution was built. In this equipment (see diagram, Fig. 6) the column and sampling sections were essentially unchanged. The reservoir was made of thick-walled Pyrex glass. All control valves were similar to the letdown valves previously described, *i.e.*, they involved plastic tubings in metal retaining sleeves which also hold the valve stem. The valves were sized somewhat larger than the letdown valve since they connect larger diameter (9 mm O.D.) capillaries. Details of the connections (A, B, C and D of Fig. 6) are shown in Fig. 7. Tygon tubing, flared at both ends, extends through C and D, connecting the column and the lower end of the reservoir. Connections B and D also serve as valves. As in the letdown valve, a nylon button is pushed by a screw against the Tygon tubing. However, to prevent extensive deformation of the plastic tubing, the valve also contains a set screw which provides a flat seat, located approximately in the middle of the opening, against which the valve operates. Since it is desirable to attach the valves to the glass tubings after the glass blowing is complete, the connectors were redesigned. They now involve a split nut which is placed over the wide portion of the glass tubing and which is held in place by a retaining nut with a hole sufficiently large to accommodate the widest part of the glass tubing.

The valve stems extend through a lucite shield which covers all but the back of the equipment. Here a glass wool filter is installed. In case of breakage it prevents dangerous pressure buildup while trapping solution and particles. This feature is especially desirable if substantial amounts of radioactive tracers are used.

With the sleeved plastic tubing as connectors it was unnecessary to install a special thrust cradle for the column. This permitted insertion of the column through the top plate and filling of the column from the top, but necessitated a redesign of the end plates. The top and bottom plates were provided with oversized holes for admission

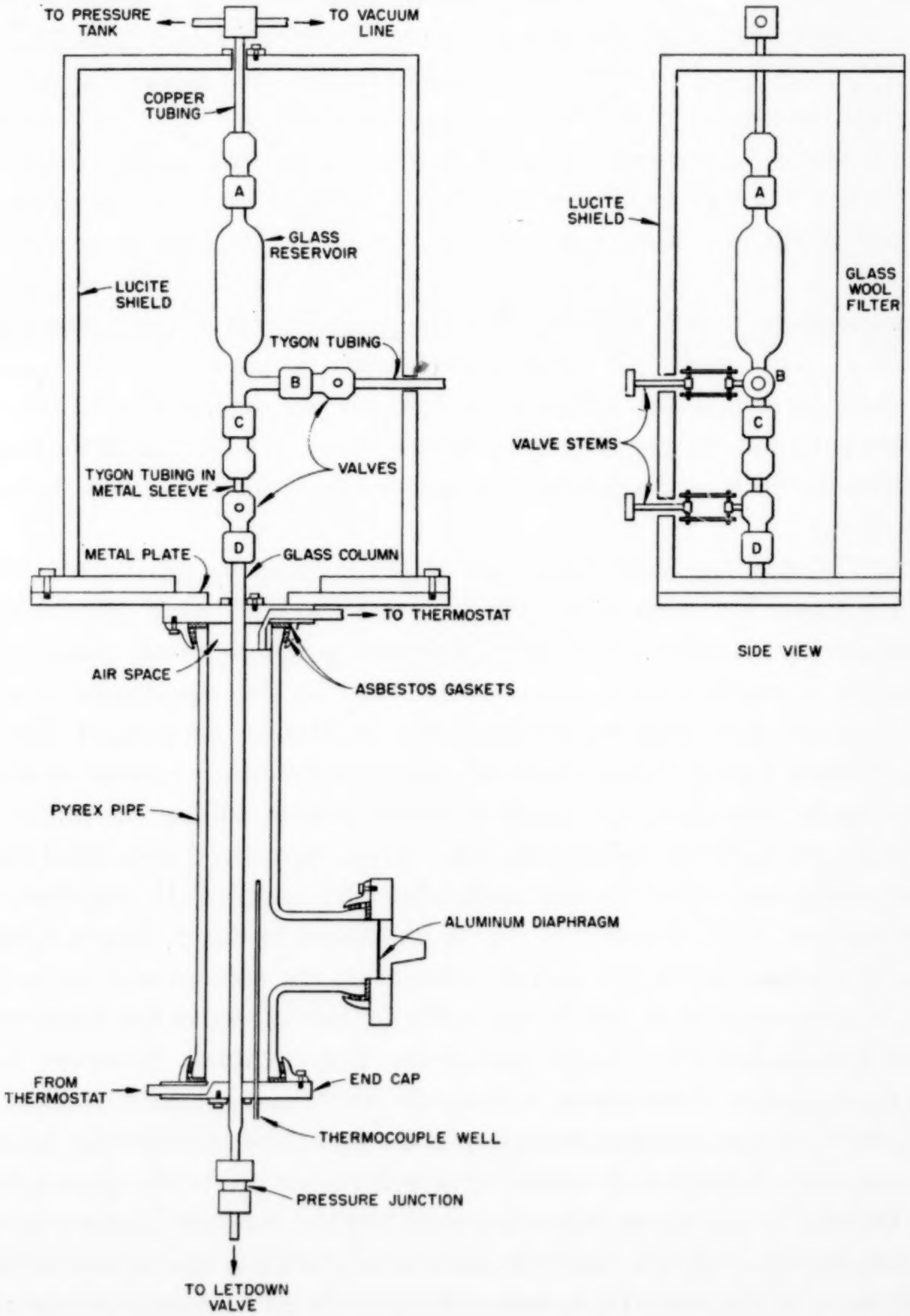
TO PRESSURE TANK
TO VACUUM LINE
COPPER TUBING
A
GLASS RESERVOIR
LUCITE SHIELD
TYGON TUBING
B
C
VALVES
TYGON TUBING IN METAL SLEEVE
D
METAL PLATE
GLASS COLUMN
TO THERMOSTAT
AIR SPACE
ASBESTOS GASKETS
PYREX PIPE
ALUMINUM DIAPHRAGM
FROM THERMOSTAT
END CAP
THERMOCOUPLE WELL
PRESSURE JUNCTION
TO LETDOWN VALVE
LUCITE SHIELD
GLASS WOOL FILTER
VALVE STEMS
SIDE VIEW

Fig. 6. High temperature ion exchange column (glass–plastic design).

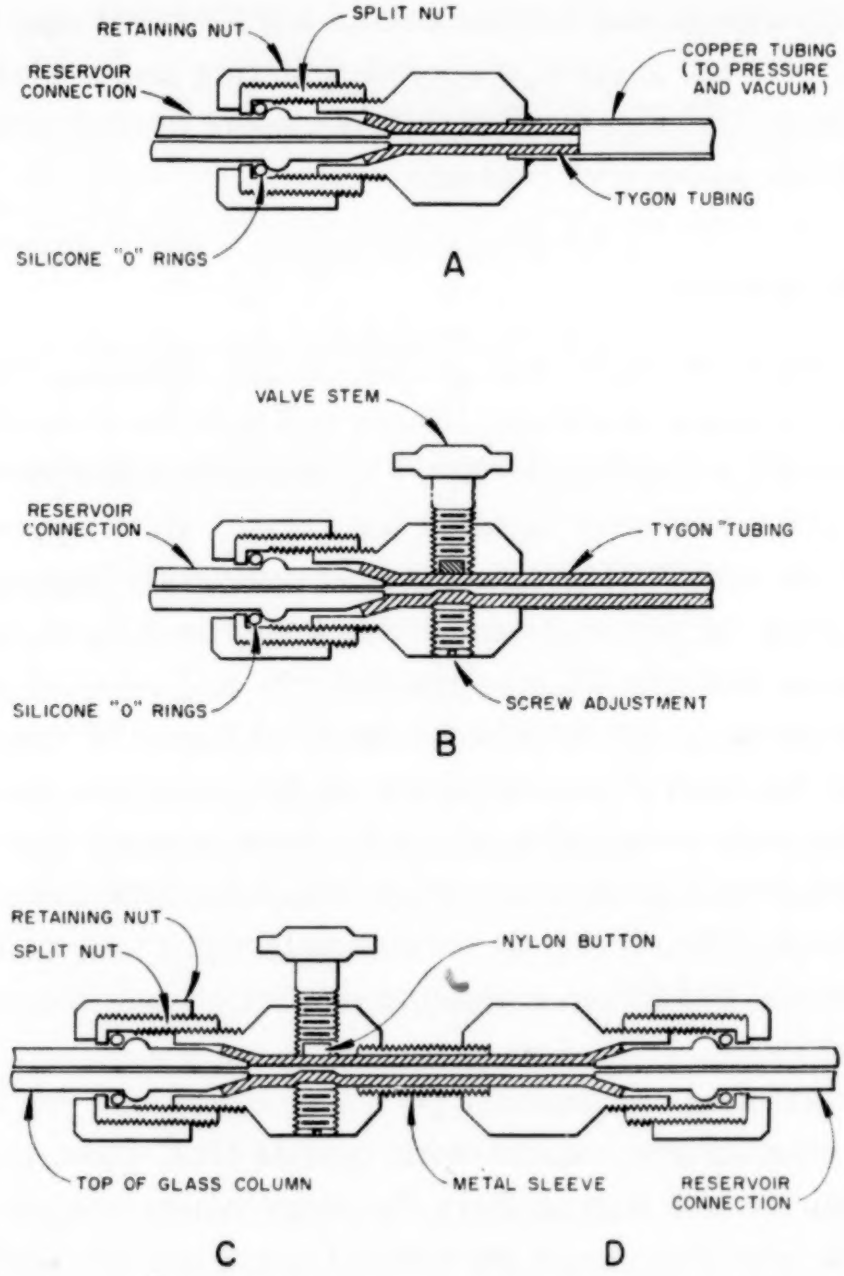
RETAINING NUT
SPLIT NUT
RESERVOIR CONNECTION
COPPER TUBING (TO PRESSURE AND VACUUM)
TYGON TUBING
SILICONE "O" RINGS
A
VALVE STEM
RESERVOIR CONNECTION
TYGON TUBING
SILICONE "O" RINGS
SCREW ADJUSTMENT
B
RETAINING NUT
SPLIT NUT
NYLON BUTTON
TOP OF GLASS COLUMN
METAL SLEEVE
RESERVOIR CONNECTION
C
D

Fig. 7. Details of connections and valves.

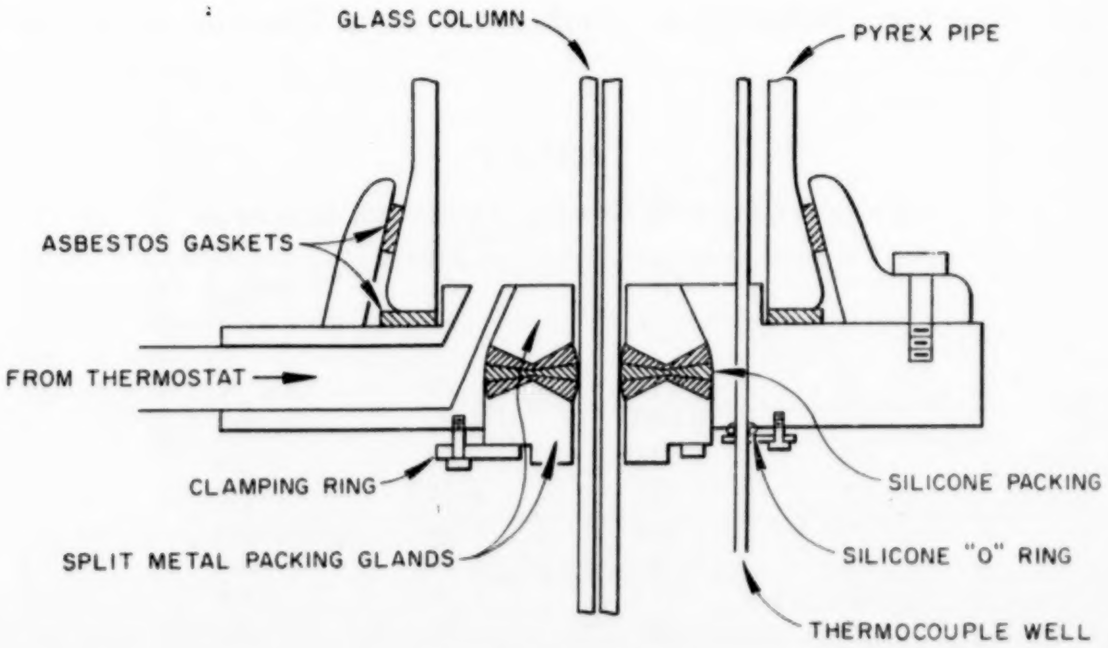
GLASS COLUMN
PYREX PIPE
ASBESTOS GASKETS
FROM THERMOSTAT
CLAMPING RING
SPLIT METAL PACKING GLANDS
SILICONE PACKING
SILICONE "O" RING
THERMOCOUPLE WELL

Fig. 8. End cap detail.

of the column. These were closed by insertion of a split metal ring followed by three silicone rubber compression gaskets, a second split ring and a plate which could be tightened with 3 screws (see Fig. 8). This type of closure proved satisfactory since the jacket in general is not under high pressure.

### 2. *Bromide–chloride exchange*

The adsorption of tracer bromide was studied in the temperature range 5 to 150° with the strongly basic anion exchanger Dowex-1-X10 in the chloride form (mesh size 170–230). The tracer (*ca.* 0.5 millicurie $^{82}$Br, $T_{1/2}$ 36 h) was adsorbed on the resin from 0.5 $M$ HCl. Resin and tracer were agitated for 2 days, which appeared sufficient for uniform loading of the sample. The tracer was of sufficiently high specific activity so that resin loading with respect to bromide was less than 1 % of exchange capacity.

The loaded resin was placed into the column and washed with 0.01 $M$ HCl. Concentration of bromide in the resin was computed from the known initial concentration of tracer in the sample (corrected for decay) and from analysis of the wash solution. To conform with our usual units, resin concentrations are expressed in terms of kilograms of dried resin. The dry-resin weight was computed from the known weight of the air-dried resin and the separately determined weight ratio of air-dried resin and resin dried (to constant weight) in a vacuum desiccator over the dehydrating agent "Anhydrone" at 60°.

Distribution coefficients $D$ (amount per kg resin/amount per kg of water) were determined by elution of the column with 0.01 $M$ HCl. Flow rate was usually 3 cm/min (c.c./cm$^2$/min) which was easily in the range where the effluent activity was independent of flow-rate. Samples of the effluent (*ca.* 1 ml) were collected in weighed glass tubes until the tracer concentration in successive samples was essentially constant. The sample size was ascertained by weighing and the bromide concentration by counting the tubes in a well-type scintillation counter (NaI-Tl). Measurements were carried out with increasing and decreasing temperature and the values, which usually agreed to better than ± 1 %, and in all cases to better than 2 %, were averaged. The observed (average) distribution coefficients as a function of temperature are listed in Table I.

TABLE I

COEFFICIENTS FOR $Br^-$–$Cl^-$ EXCHANGE REACTION

| $t$ (°C) | $D$ | $K'$ | $K'_{calc}$ |
|---|---|---|---|
| 5 | 1350 | 3.70 | 3.71 |
| 25 | 1120 | 3.07 | 3.06 |
| 50 | 923 | 2.52 | 2.52 |
| 75 | 789 | 2.16 | 2.17 |
| 100 | 704 | 1.92 | 1.93 |
| 125 | 645 | 1.76 | 1.75 |
| 140 | 623 | 1.70 | 1.68 |
| 150 | 590 | 1.61 | 1.63 |

The mass action expression for the ion exchange equilibrium may be written as

$$K \equiv 1 = \frac{m_{\mathrm{Br}(r)}\, m_{\mathrm{Cl}}}{m_{\mathrm{Br}}\, m_{\mathrm{Cl}(r)}} \frac{\gamma_{\mathrm{Br}(r)}\, \gamma_{\mathrm{Cl}}}{\gamma_{\mathrm{Br}}\, \gamma_{\mathrm{Cl}(r)}} = K'\Gamma_{(r)} \qquad (1)$$

where:

$m$ = concentrations

$\gamma$ = activity coefficients of ions

$K' = \dfrac{m_{\mathrm{Br}(r)}\, m_{\mathrm{Cl}}}{m_{\mathrm{Br}}\, m_{\mathrm{Cl}(r)}} \dfrac{\gamma_{\mathrm{Cl}}}{\gamma_{\mathrm{Br}}}$ = the selectivity coefficient corrected for the activity coefficients in the aqueous phase

$\Gamma_{(r)} = \dfrac{\gamma_{\mathrm{Br}(r)}}{\gamma_{\mathrm{Cl}(r)}}$ = the activity coefficient ratio of the ions in the resin phase.

As is customary in this laboratory, we have taken the same standard states for the ions in the resin and aqueous phases and hence have set $K \equiv 1$ at all temperatures.

Since the electrolyte solutions used were very dilute we have assumed $\gamma_{\mathrm{Cl}}/\gamma_{\mathrm{Br}} = 1$ and hence computed $K'$ from the observed distribution coefficients ($D = m_{\mathrm{Br}(r)}/m_{\mathrm{Br}}$) and the chloride concentration of the solutions ($m_{\mathrm{Cl}}$). The chloride concentration in the resin ($m_{\mathrm{Cl}(r)}$) was taken to be equal to the capacity of the resin (3.66 equivalents per kg) since in these dilute solutions resin invasion by the electrolyte is negligible.

As shown in Table I, $K'$ decreases only moderately with temperature from 3.70 at 5° C to 1.61 at 150° C. No measurements at higher temperatures have been recorded since the results tended to be erratic, probably a result of slow decomposition of the resin.

A plot of log $K'$ *vs.* $1/T$ (Fig. 9) (where $T$ is the absolute temperature) shows slight curvature, implying that the heat of adsorption $\Delta H'$ of bromide tracer on the chloride form of the resin is not constant. We have fitted the observed data to the empirical equation

$$\log K' = \log K'_t + \alpha \log (T/T_t) + \beta\, [1 - (T_t/T)] \qquad (2)$$

where $\alpha$ and $\beta$ are constants and subscript "$t$" refers to a reference temperature, here taken as 75° C. This equation is derived from the assumption that the heat capacity

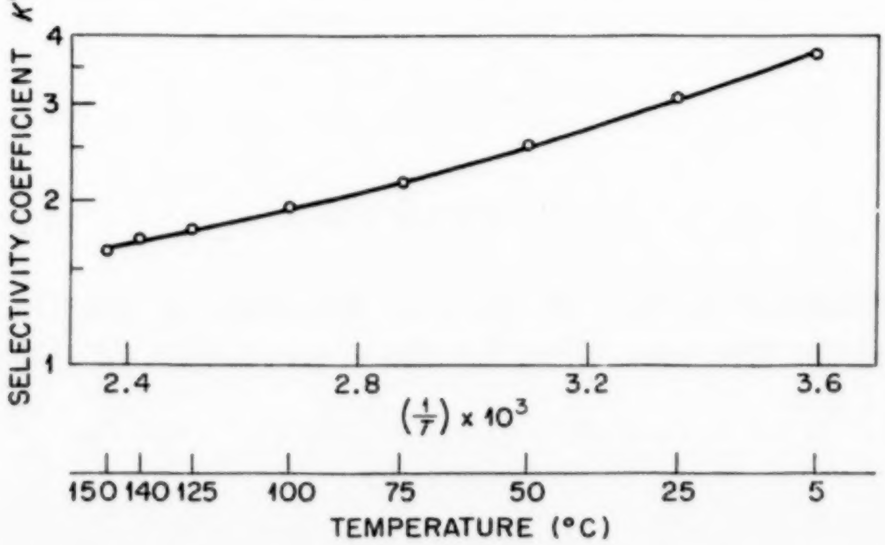

Fig. 9. Temperature dependence of anion exchange equilibria (bromide tracer on Dowex-1-X10, Cl⁻ form).

change on adsorption, $\Delta\bar{C}'_p$, is constant. Analysis of the data gave $\alpha = 2.500$, $\beta = -1.893$, and $\log K'_t = 0.336$. The solid curve in Fig. 9 is computed with these parameters and more detailed comparison between calculated and observed values of $K'$ is included in Table I. The small scatter of observed values of $K'$ and those calculated by eqn. (2) is noteworthy, since it further demonstrates the high inherent precision of the method.

Through differentiation of eqn. (2) we obtain

$$\Delta\bar{H}' = -R\,\mathrm{d}\ln K'/\mathrm{d}(1/T) = aRT + 2.303\,\beta RT_t \qquad (3)$$

$$\Delta\bar{C}'_p = \mathrm{d}(\Delta\bar{H}')/\mathrm{d}T = aR \qquad (4)$$

and

$$\begin{aligned}\Delta\bar{S}' &= (\Delta\bar{H}' - \Delta\bar{F}')/T \\ &= aR + 2.303\,\beta RT_t/T + R\ln K' \qquad (5) \\ &= aR + 4.576\,\beta + R\ln K'_t + aR\ln(T/T_t)\end{aligned}$$

through which the partial molal enthalpy, heat capacity and entropy changes for the adsorption reaction may be computed from the parameters $\alpha$, $\beta$ and $K'_t$. Values of $\Delta\bar{H}'$, $\Delta\bar{S}'$ and of $(-RT\ln K')$ computed at round temperatures and extrapolated to 200° C are given in Table II. The heat capacity change $\Delta\bar{C}'_p$ for the reaction was calculated to be 5.0 cal/°C and $T_{\mathrm{min}}$, where the fitting function has a minimum, is 607° K.

TABLE II

PARTIAL MOLAL ENTHALPY AND ENTROPY CHANGES FOR $Br^-$–$Cl^-$ EXCHANGE REACTION

| $t$ (°C) | $-RT\ln K'$ (kcal) | $\Delta\bar{H}'$ (kcal) | $\Delta\bar{S}'$ (e.u.) |
|---|---|---|---|
| 0 | −0.74 | −1.66 | −3.4 |
| 25 | −0.66 | −1.53 | −2.9 |
| 50 | −0.59 | −1.41 | −2.5 |
| 75 | −0.54 | −1.29 | −2.2 |
| 100 | −0.49 | −1.16 | −1.8 |
| 125 | −0.44 | −1.04 | −1.5 |
| 150 | −0.41 | −0.91 | −1.2 |
| 175 | −0.39 | −0.79 | −0.9 |
| 200 | −0.37 | −0.67 | −0.6 |

## ACKNOWLEDGEMENT

We are greatly indebted to Mr. W. L. VAN HOOZIER of the Oak Ridge National Laboratory Chemistry Division Shop for the construction of the high temperature columns.

## SUMMARY

A packed column method for the precision measurement of temperature coefficients of ion exchange equilibria has been developed and equipment suitable for operation up

to 200° is described. Use of the equipment is illustrated with the bromide (tracer)–chloride anion exchange system in the range 0° to 150° for which the enthalpy change was found to be a linear function of temperature.

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# SEPARATION AND IDENTIFICATION OF CAFFEINE, ANTIPYRINE AND PHENACETIN FROM HUMAN TISSUE

R. BONNICHSEN, A. C. MAEHLY AND S. NORDLANDER
*Government Laboratory for Forensic Chemistry, Stockholm (Sweden)*

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Upon extraction of acidified tissue homogenates with organic solvents, neutral and acidic compounds of various kinds are obtained. Among these substances barbiturates are of special toxicological interest. In the course of barbiturate analyses performed in this laboratory, it was found that three other compounds, caffeine, antipyrine (phenazone), and phenacetin (acetophenetidine), which are extracted together with the barbiturates, can be easily separated, identified and assayed at the same time as the barbiturates with the help of paper chromatography.

Separation of these three compounds from each other has been achieved by SJÖSTRÖM[1], using ion exchange columns; similar methods for the separation of one or two of these substances from each other and from contaminants have been reported. Our standard procedure is as follows: The tissue to be analyzed (as a rule 30 g portions of either liver, kidney, blood or urine) is acidified to pH 3 and extracted with chloroform, both before and after acid hydrolysis. After evaporation to dryness, the residue is freed from fat by treatment with very dilute warm acid and filtration in the cold. The filtrate is extracted with chloroform. Strongly acidic compounds are removed from the chloroform extract with phosphate buffer of pH 7.2. Caffeine, antipyrine, and phenacetin are almost quantitatively transferred to the final chloroform extract. The distribution coefficients for the three compounds are listed in Table I. The "washed" chloroform extract is now analyzed by descending chromatography on Whatman No. 1 paper (25 × 50 cm) that has been treated with 0.05 $M$ $Na_2CO_3$ solution. Standard solutions of the 10 most common barbituric acid derivatives are used as controls on the same sheet. Water-saturated chloroform, diethyl ether or di-*n*-butyl ether are used as the mobile phase. The running time is about 1.5 hours for

TABLE I

DISTRIBUTION COEFFICIENTS BETWEEN CHLOROFORM AND WATER*

| *Chloroform* | *Caffeine* | *Antipyrine* | *Phenacetin* |
|---|---|---|---|
| 0.1 $N$ HCl | 16.5 | 5.5 | 28.4 |
| Phosphate buffer pH 7 | 24.6 | 17.5 | > 100 |
| 0.5 $N$ $NH_4OH$ | 16.5 | 26.8 | 32.4 |

* Ratio of concentrations of the solute in chloroform and in the aqueous phase, using equal volumes of each solvent.

ethyl ether, about 2 hours for chloroform, and 5 hours for *n*-butyl ether. In the last case, the solvent front is allowed to leave the paper.

Paper chromatography of barbiturates is usually carried out with chloroform as the mobile phase[2], though butanol or amyl alcohol have also been employed. Table II shows that with chloroform as a solvent the three compounds discussed here all move close to the solvent front, and thus are apt to be lost in tissue impurities. But with ether as the moving phase, separation is excellent. However, these compounds can be confused with certain barbiturates: caffeine and phenobarbital have very similar $R_F$ values and so have antipyrine and vinbarbital as well as phenacetin and pentobarbital. Only subsequent analysis will reveal the true nature of the compound. Antipyrine can further be detected by spraying with Dragendorff's reagent or ferric chloride.

After completion of the run, the papers are air-dried, placed on a paper previously treated with a solution of 0.005% fluorescein in 0.5% ammonia, and the "spots" located in ultraviolet light ("Mineralite" of wavelength 254 mμ)*. The $R_F$ values for caffeine, antipyrine, and phenacetin are listed in Table II. The "spots" are eluted from the paper and assayed spectrophotometrically. Paper blanks of similar size are treated in the same way. The eluates from the "spots" are evaporated and dissolved in ethanol–0.5 *M* ammonia (3:1). The spectra of the eluted substances are read both before and after acidification with hydrochloric acid, using the solution of the paper extracts as a blank. The pertinent absorption maxima and minima in the ultraviolet region are listed in Table II. The yields are very satisfactory. When 0.50 mg each of caffeine, antipyrine, and phenacetin were added to 30 g of human liver and the above

TABLE II

SOME PHYSICAL PROPERTIES OF CAFFEINE, ANTIPYRINE, AND PHENACETIN

(a) Stands for acid pH, (b) for alkaline pH, and (n) for neutral pH

| | *Caffeine* | *Antipyrine* | *Phenacetin* |
|---|---|---|---|
| $R_F$ (chloroform) | 0.95 | 0.95 | 0.95 |
| $R_F$ (ether) | 0.41 | 0.55 | 0.90 |
| $R_F$ (*n*-butyl ether)* | 0.09 | 0.13 | 0.75 |
| Ultraviolet maxima (mμ)** | 272 (b) 270 (a)<br>*E* 420 — | 244 (b and n) 270 (b and n) 236 (a)<br>*E* 488 *E* 480 — | 250 (b, n and a)<br>*E* 822 |
| Ultraviolet minima (mμ) | 244 (b, n and a) | 256 (b and n) | — |
| Infrared maxima (cm⁻¹)*** | $\underline{1703}$, $\underline{1660}$, 1548, 1484, 1238, 1023, 970, 738 | $\underline{1685}$, 1598, 1494, 1393, 1332, 1134 | 3270, $\underline{1658}$, 1553, 1511, 1482, 1243, 1043, 834 |
| Melting point | 238° (subl.) | 111–113° | 134–135° |

* 5 hours running time.
** *E* = extinction coefficient for 1% solution and 1 cm lightpath.
*** Intense bands underlined.

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* Details of the analytical procedure for barbiturates will be published elsewhere[3].

procedure carried out, over-all yields of 82.7 % and 83.9 % for caffeine, 79.6 % and 79.8 % for antipyrine, and finally 97.3 % and 99.6 % for phenacetin were obtained.

In actual toxicological cases, especially when kidney and urine are extracted, varying amounts of metabolites are found on the chromatograms along with the unaltered compounds[4, 5]. In these cases additional criteria such as infrared spectra and melting point determination are needed for final identification. Filter paper itself contains so much chloroform-soluble foreign material, that the compounds must be subjected to sublimation before satisfactory melting points or infrared spectra can be taken.

Infrared spectra are obtained in the following way. The eluted material containing 10–50 $\mu$g of the compound is transferred to a microbeaker, covered with a thin potassium bromide disk (0.5 inch diameter) and placed on the stage of a Kofler block-equipped microscope. The material is sublimated upon the potassium bromide disk and analyzed in the reflecting microscope of a Hilger H 800 infrared spectrophotometer.

Out of about 500 autopsy cases investigated in the past two years, antipyrine was found in 21 cases, phenacetin in 20 cases, and caffeine in 53 cases. About half of the analyses were carried out on a quantitative basis, and up to 8.0 mg antipyrine, 4.0 mg phenacetin, and 1.6 mg caffeine were found per 100 g of tissue. In cases where coffee had been consumed some caffeine is usually found, and sometimes enough theobromine is present to be detected on the chromatogram. With ether as the mobile phase theobromine stays close to the origin, while with chloroform the $R_F$ value is about 0.22.

## ACKNOWLEDGEMENT

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## SUMMARY

Separation of caffeine, antipyrine and phenacetin from human tissue by paper chromatography is described. The compounds are eluted from the paper and identified by their ultraviolet spectra and by infrared micro spectrophotometry. Methods for quantitative determination are given.

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# Short Communications

## Paper chromatography of inorganic ions in nitrate solutions

## I. Scandium, yttrium, actinium and the lanthanides

The separation of the rare earths by paper chromatography in nitric acid media and a number of organic solvents was investigated by LEDERER[1]. Under these conditions a slight difference between the individual $R_F$ values was observed, but no separation could be achieved since considerable tailing occurred.

Analogous results were obtained in anion-exchange studies of the rare earths in $HNO_3$ media[2]. These elements are adsorbed to a slight extent by Dowex-1 from concentrated $HNO_3$ solutions, but the small differences between the values of the distribution coefficients do not allow efficient separations.

Further studies have shown that the adsorption of these elements by the resin is considerably enhanced when a soluble nitrate such as lithium nitrate is added to the $HNO_3$ solution[3]. Successful separations of Ac and La[3] and of the individual lanthanides[4] on Dowex-1 were obtained in this medium.

On the basis of these results we investigated the separation of Sc, Y, Ac and the lanthanides by paper chromatography in $LiNO_3$ solution. Since the rare earths are extracted by alcohols from concentrated nitrate solutions[5] we used this kind of solvent in our investigations. The results obtained in a typical experiment are given in Table I. These data were obtained by descending development during 72 hours of a chromatogram in butanol previously saturated with a solution 7 $M$ $LiNO_3$–2 $M$ $HNO_3$, at room temperature (25° ± 3°). The rare earths were detected with 8-hydroxyquinoline followed by examination of the fluorescence of the spots in ultraviolet light. The $R_F$ values obtained *under these conditions* are referred to the second (dark) front of the solvent.

TABLE I

| *Element* | $R_F$ | *Element* | $R_F$ | *Element* | $R_F$ |
|---|---|---|---|---|---|
| La | 0.40 | Sm | 0.55 | Er | 0.64 |
| Ce | 0.46 | Eu | 0.55 | Y | 0.58 |
| Pr | 0.51 | Gd | 0.56 | Sc | 1.00 |
| Nd | 0.54 | Dy | 0.62 | | |

In Fig. 1 a chromatogram obtained with La, Y and Sc under the same conditions is reproduced.

The separation of trace amounts of actinium from lanthanum was investigated with $^{228}$Ac (MsTh II, $\beta$, $\gamma$, half-life 6.13 hours). This radioelement was extracted from a $^{228}$Ra source (MsTh I) and its purity was checked by half-life period measurements in a scintillation counter. The results obtained with a development period of 30 hours are shown in Fig. 2.

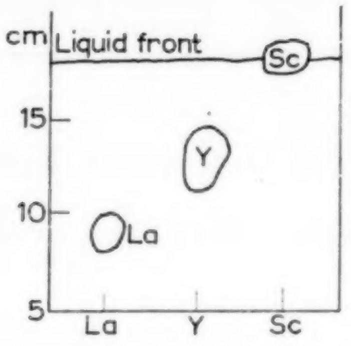

Fig. 1.

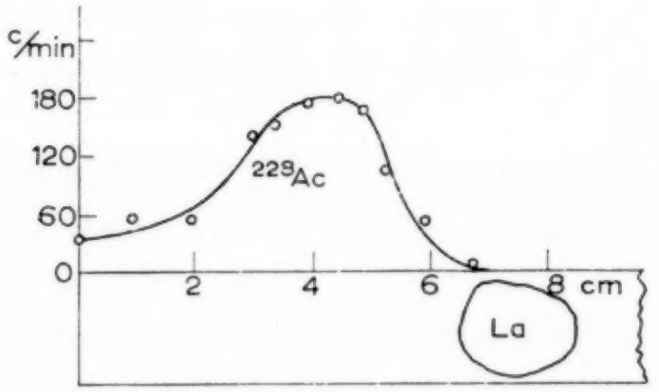

Fig. 2.

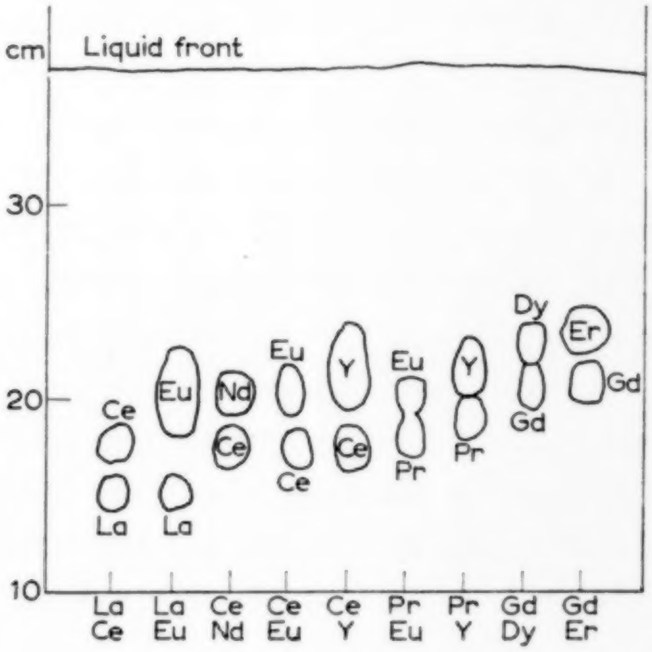

Fig. 3.

Fig. 3 illustrates a number of separations of the lanthanides.

The results given in Table I demonstrate that the experimental conditions are not suitable for the separation of adjacent lanthanides, except for La, Ce and Pr. No improvement in the separations was obtained by using other solvents such as ethyl, propyl, amyl and iso-amyl alcohol or by developing the chromatograms at higher temperatures.

*Brazilian Centre for Physics Research**, *Rio de Janeiro* (*Brazil*)
J. Danon
M. C. Levi

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* Centro Brasileiro de Pesquisas Físicas, Avenida Wenceslau Braz 71, Rio de Janeiro.

# Metallic nitrates in paper chromatography

## VI. Systems containing tri-*n*-butyl phosphate and nitric acid*

Continuing our studies[1-5] on the chromatographic behaviour of metallic nitrates in those organic solvents that are used for extraction in radiochemistry, we have recently investigated the usefulness of tri-*n*-butyl phosphate (TBP) as an eluant in partition chromatography.

TBP has undoubtedly become one of the most favoured organic solvents for the separation of fissile and fertile materials. The extraction of the actinides and rare earths by TBP and the conditions for the separation of these elements from one another have been extensively studied[6]. A chromatographic separation of nickel from cobalt by TBP in hydrochloric acid media has recently been reported[7].

Since partition chromatography provides results that are related to the distribution coefficients obtained by the extraction technique, we investigated the chromatographic behaviour of the 59 cations (with the exception of osmium) and anions listed in the preceding paper[5]. In addition to the ions known to form complex species that can be extracted from nitric acid solutions by TBP, it was found that the following ions moved on the paper with appreciable $R_F$ values: calcium, magnesium, aluminium, beryllium, indium, manganese, selenium, rhenium, iron, molybdenum, vanadium, technetium, bismuth, zinc and iodine. Furthermore, the covalent chloride compounds of antimony, tin and palladium were appreciably extracted by TBP.

During the first preliminary experiments, several technical difficulties were encountered. The TBP solvent continued to move along with the spots on the paper even after the run of the chromatograms had ceased, which necessitated an immediate developing of the chromatograms. Further difficulties arose in the developing of the spots, owing to the stable metallic complexes formed with the TBP. The most serious disadvantage was the tailing of the spots on the paper. In order to overcome the difficulties encountered, nearly all the factors that influence the chromatographic behaviour of ions were investigated. Experiments were carried out varying: (1) the technique employed, (2) the initial concentration of nitric acid and/or of inorganic nitrates, (3) the organic diluents and (4) the concentration of TBP in the organic diluent.

Both the ascending and descending techniques were employed. By using longer sheets of paper in the descending technique we succeeded in shortening the tailing appreciably and in obtaining well defined spots. Some experiments were carried out using paper impregnated with TBP. Only a very slight migration of the ions ($R_F \leqslant 0.1$) occurred on these papers, independent of the nitric acid concentration.

Whatman papers Nos. 1, 2, 3 and 4 were compared, using the radioactive tracer

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[*] A preliminary note. Part of a thesis to be submitted by A. BECK to the Senate of the Hebrew University of Jerusalem in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

technique. In this way the difficulties encountered in the developing of the chromatograms were eliminated. Whatman No. 4 was found to be the most suitable in most cases, since it gave well defined spots.

The $R_F$ values of most of the ions are not influenced when the nitric acid concentration is varied between 1 and 16 $M$, although for some cations a minimum concentration of the acid is necessary to obtain $R_F$ values other than zero. The type of nitrate (lithium, sodium or ammonium nitrate) in the aqueous solution also had no marked influence on the $R_F$ values measured.

In practice TBP is diluted with an inert solvent, usually kerosene. Our experiments were carried out with 0%, 5%, 20% and 50% TBP, and also with 100% TBP; di- and mono-butyl phosphate had previously been removed from the TBP[8]. In solutions containing 0% and 5% TBP, all the ions investigated had $R_F$ values equal to zero. An increase of the TBP content from 20% to 100% caused only a slight increase in the $R_F$ values. With 100% TBP the tailing of the ions was less in several cases; when the descending technique was employed quite well defined spots were obtained.

Purified and redistilled odourless kerosene boiling between 210°–250° was used in most of the experiments. Instead of kerosene, carbon tetrachloride, chloroform, heptane, toluene, petroleum ether, butanol and methyl isobutyl ketone were also tried. None of these solvents showed any great advantages over kerosene. When butanol or methyl isobutyl ketone were used as diluents, double-spots appeared in several cases, probably due to the diluents themselves being capable of extracting the ions.

Summarizing, it can be said that the number of ions that migrate on the paper is significantly larger than the number that up till now have been reported to be extractable from aqueous nitric acid solutions by TBP. Neither the amount of TBP in the diluent, nor the nitric acid concentration of the aqueous phase had an appreciable influence upon the $R_F$ values obtained This is in contrast to what was observed in the case of the extraction technique.

A more detailed report, including numerical data for the ions investigated, will be published in due course.

*Department of Inorganic and Analytical Chemistry,*
*The Hebrew University of Jerusalem (Israel)*

A. S. KERTES
A. BECK

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# A paper-chromatographic method for the identification of α- and β-naphthol when present together

α-Naphthol and β-naphthol are constituents of high-boiling coal tar fractions and of the two the latter is especially important for it is the starting material for a wide range of synthetic dyes and their intermediates[1]. Several methods for the detection of α- and β-naphthol when they are present together have been suggested. One such method is based on the fact that the phenyl-azo derivative of α-naphthol is soluble in water, whereas that of β-naphthol is insoluble[2]. Methods based on the chromatography of naphthols or of their derivatives have also been proposed[3]. EVANS *et al.*[4] obtained paper chromatograms of free naphthols using a mixture of butanol, pyridine and sodium chloride solutions as developing agent and a solution of diazotised sulphanilic acid for the detection. Although the spots of the two phenols are somewhat different in colour their $R_F$ values are identical, thus making it difficult to distinguish between them. RILEY[5] used *n*-amyl alcohol saturated with water or *n*-butanol–benzene–water for developing the spots and detected them by spraying with phosphomolybdic acid. With both solvents the $R_F$ values of the two phenols were nearly equal. BARTON, EVANS AND GARDNER[6] used $H_2CO_3$ solution for the development of the chromatograms and $FeCl_3$ or $K_3Fe(CN)_6$ as spraying reagents for the detection of the phenols.

In a series of papers, HOSSFELD *et al.* (see *e.g.*[7]) have attempted to identify phenols by coupling them with diazotised sulphanilic acid and then chromatographing the resulting dye. They used this technique for the identification of several phenols, including the naphthols. In the case of phenols whose derivatives have almost identical $R_F$ values, the technique of two-dimensional chromatography was applied for affecting the separation.

We were interested in developing paper chromatographic methods[8] and during our investigations we observed that dyes lend themselves to easy separation if a suitable developing agent is used. Some of the phenols[9] can be identified by paper chromatographic separation of their diazo derivatives. We therefore thought that it would be possible to develop a method for the identification of phenols, particularly the naphthols, which would involve the preparation of diazo derivatives of the phenols followed by paper chromatographic development of these derivatives.

Amino-J acid (disodium salt of 2-naphthylamine-5,7-disulphonic acid) on diazotisation and coupling with the various phenols gave distinctly coloured spots on filter paper, which could be developed with a suitable eluent. Especially the results obtained with α- and β-naphthol were encouraging because the $R_F$ values obtained with certain developing agents differed appreciably for the two naphthols. The following method was, therefore, developed for the identification of α- and β-naphthol when present together in a mixture.

*Experimental.* 0.1% solutions of α- and β-naphthol were prepared separately

TABLE I

$R_F$ VALUES OF DYES OBTAINED BY COUPLING DIAZOTISED AMINO-J ACID WITH $\alpha$- AND $\beta$-NAPHTHOL

| *Eluent* | *$R_F$ value* | |
|---|---|---|
| | *Dye from α-naphthol* | *Dye from β-naphthol* |
| 1. Isoamyl alcohol–ethyl alcohol–ammonia (40:40:25) | 0.14 | 0.28 |
| 2. Isoamyl alcohol–ethyl alcohol–water (40:40:25)* | 0.10 | 0.24 |
| 3. Methyl isobutyl ketone–ethyl alcohol (70:30) saturated with ammonia (10%) | 0.04 | 0.18 |

* Paper impregnated with 3% caustic soda solution before applying the spots.

in alcohol and one drop of each solution was placed about 2 inches apart on the base line of Whatman filter paper No. 1. The spots were coupled *in situ* with a cold alkaline solution of diazotised Amino-J acid (approx. $M/50$). A dye was immediately formed. The paper was allowed to dry, rolled into the form of a cylinder and placed in a trough containing the desired eluent and then covered immediately with a bell jar. The usual precautions of saturating the atmosphere inside the bell jar and sealing the flange of the bell jar with silicone grease after assembly, were taken. The chromatogram was developed until the liquid front had moved up about 7–8 inches. This took nearly 4 hours. The spots of the dye formed from α- or β-naphthol also moved up. The $R_F$ values of these dyes with different developing agents are recorded in Table I.

The colour of the spots, when wet, were rose-red for α-naphthol and reddish orange for β-naphthol in the case of all three eluents. On drying, the spots obtained

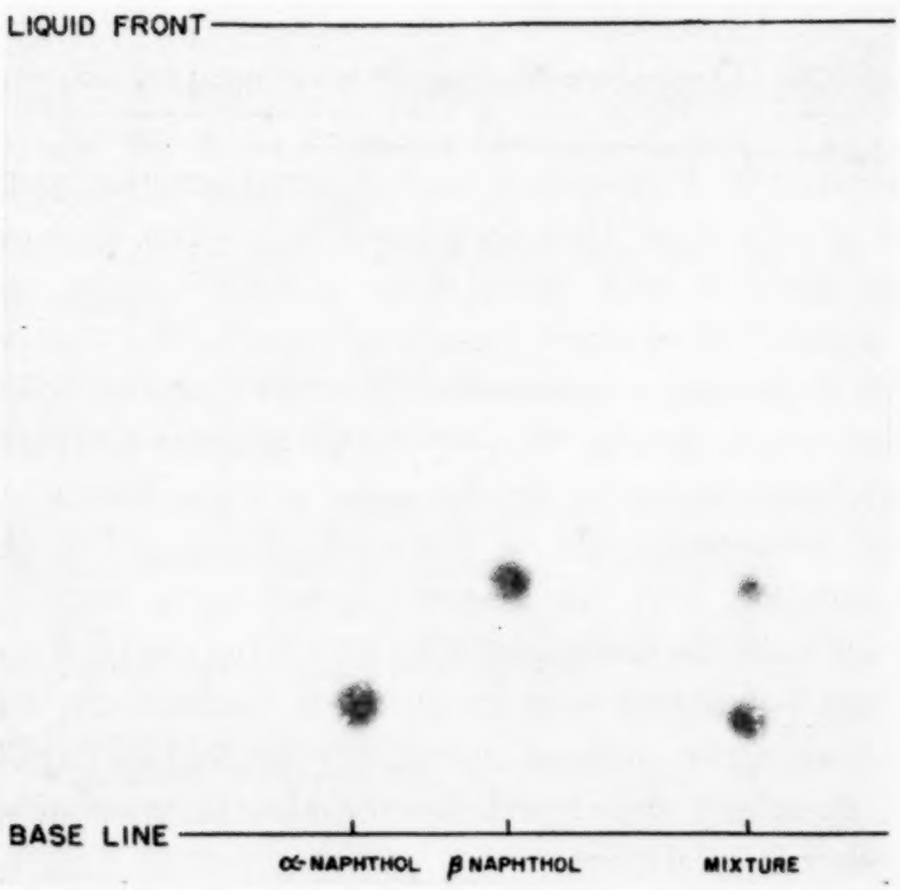

Fig. 1.

with eluents (1) and (3) acquired the same rose red colour; in the case of eluent (2) the dye spots retained their original shade.

It can be seen from Table I that the $R_F$ values for the α- and β-naphthol derivatives differ considerably in all three systems. In a separate experiment, one drop of a solution containing both α- and β-naphthol was placed alongside the two drops of the α-naphthol and β-naphthol solutions. After coupling all three spots separately with diazotised Amino-J acid, the paper was dried and developed with isoamyl alcohol–ethyl alcohol–ammonia solution. A typical chromatogram is shown in Fig. 1. It can be seen that on development the spot of the mixture of the two naphthols is separated into two spots, which move parallel to those of α- and β-naphthol respectively.

*National Physical Laboratory, New Delhi (India)*
M. R. VERMA
RAMJI DASS
P. K. GUPTA

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## Quantitative determination of proline by paper chromatography

Since the ninhydrin reagents are not very sensitive to proline, the isatin method for the detection of this amino acid has frequently been used, even for quantitative purposes. PASIEKA AND MORGAN[1] used the isatin reagent according to the method of ACHER *et al.*[2]. As these authors were not able to elute the coloured product obtained from proline with water or with other mineral or organic solvents from paper, they evaluated the spots—after washing out other spots and the background—by measuring the density of the proline areas in the cuvettes of a spectrophotometer. Since it is well known that densitometric methods *in situ* are subject to inaccuracies, it is to be expected that higher precision could be obtained if it were possible to elute the dye and measure its density in solution.

For the purpose of detecting proline on chromatograms the reagent according to ACHER *et al.*[2] and the reagent according to BARROLLIER *et al.*[3], containing Zn acetate and acetic acid, were compared. The latter is more sensitive and therefore it was used in further work. The coloured reaction product of proline can be eluted from the chromatogram—after washing out the background with water and drying—with pyridine or with alcoholic or aqueous phenol. The best results as regards intensity and stability of the eluate were obtained by using water-saturated phenol for elution. On measuring the spectra of this eluate with the spectrophotometer SF 4, maximum absorption was obtained at 610 mμ (Fig. 1).

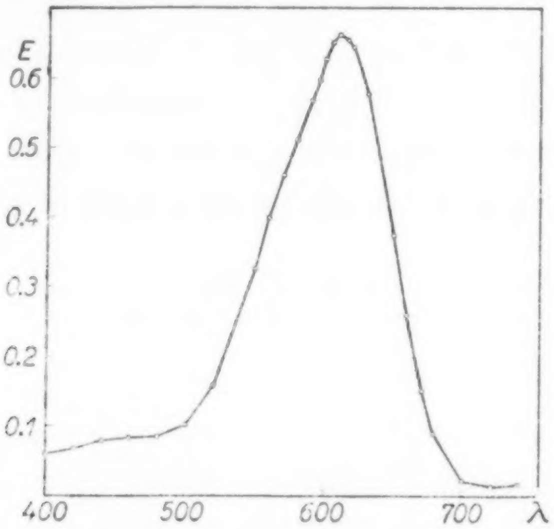

Fig. 1. Absorption curve of the coloured reaction product of proline (28.75 μg) in water-saturated phenol. Measurements made with the spectrophotometer SF 4.

A study was then made of the relation between the extinction and the amount of proline, to find out in which range it is in accordance with Lambert-Beer's law. Varying quantities of proline (ranging from 10–50 μl of 0.005 *M* proline solution) were applied to Whatman No. 1 paper and developed three times with the solvent system *n*-butanol–acetic acid–water (4:1:5) for a total period of 108 hours, which is approximately the time needed for a good separation of proline in an extract from biological material. The proline was then detected and the spots were eluted according to the method described below. The eluates were measured in the Lange photocolorimeter with the Schott OG 2 filter. The standard curve obtained (Fig. 2) showed that linearity is maintained over the concentration range up to 20 μg of proline.

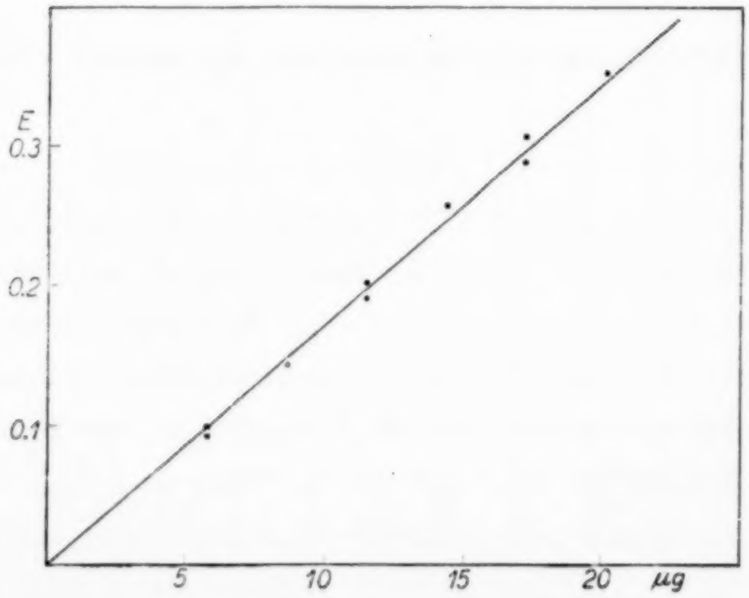

Fig. 2. Standard curve for proline. Measurements made with the Lange photocolorimeter.

The stability of the dye in water-saturated phenol in the light and in the dark was studied. The results (Table I) indicated that the eluates are stable for 1 hour in the dark but normal daylight causes a rapid destruction of the coloured proline product.

On the basis of the results obtained in these experiments, the following method for the quantitative estimation of proline by paper chromatography can be recommended.

For paper chromatography amounts of extracts of biological material containing from 5 to 20 μg of proline should be used. It is also necessary to apply at least two concentrations of the standard solution of proline to every chromatogram. After good separation of proline by a suitable solvent system, the spots on the chromatogram are detected by dipping it in the isatin reagent prepared according to BARROLLIER *et al.*[3] (1 g isatin, 1.5 g Zn acetate, 1 ml acetic acid, 95 ml isopropyl alcohol, 5 ml water) and heating for 30 min at 80–85°. The excess of isatin is removed by washing the chromatogram with warm water (about 30°), so that the background is white or pale yellow. The blank value in both cases is insignificant. After cutting the spots into small pieces, 5 ml of water-saturated phenol is added and the dye is eluted for 15 min in the dark with occasional shaking. Measurement of the density is performed at 610 mμ without unnecessarily exposing the samples to light. The accuracy of this method is ± 2.5 %.

TABLE I

STABILITY OF THE COLOURED REACTION PRODUCT OF PROLINE (23 μg) IN WATER-SATURATED PHENOL

Measurements made with the Lange photocolorimeter.

| *Time in min* | *Extinction* | | | |
|---|---|---|---|---|
| | *when exposed to light* | | *when kept in the dark* | |
| 15 | 0.433 | 0.435 | 0.462 | 0.455 |
| 30 | 0.429 | 0.433 | 0.470 | 0.456 |
| 45 | 0.423 | 0.427 | 0.468 | 0.460 |
| 60 | 0.383 | 0.385 | 0.462 | 0.453 |
| 90 | 0.340 | 0.333 | 0.450 | 0.443 |
| 120 | 0.322 | 0.326 | 0.448 | 0.438 |
| 180 | 0.268 | 0.249 | 0.425 | 0.418 |
| 300 | 0.219 | 0.221 | 0.391 | 0.387 |

*Institute of Biology, Czechoslovak Academy of Science,*
*Prague (Czechoslovakia)*

EVA HRABĚTOVÁ
JAROSLAV TUPÝ

[1] A. E. PASIEKA AND J. F. MORGAN, *Proc. Soc. Exptl. Biol. Med.*, 93 (1956) 54.
[2] R. ACHER, C. FROMAGEOT AND M. JUTISZ, *Biochim. Biophys. Acta*, 5 (1950) 81.
[3] J. BARROLLIER, J. HEILMAN AND E. WATZKE, *Z. physiol. Chem.*, 304 (1956) 21.

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## An equilibration device for paper chromatography

Equilibration of paper with solvent vapour is frequently a critical requirement for the success of a paper chromatographic separation. In many laboratories, however, where specialised chromatography tanks are not available, the techniques of equilibration in general use may be inconvenient or unsatisfactory. Thus one of the methods employed involves boring a hole in the side of the tank, from which more solvent can be added until the level of the paper is reached, whilst a common practice depends on removing the cover so as to enable a frame holding the paper to be lowered into the solvent. This communication describes a simple and efficient device for equilibration that can be made readily in the workshop to fit the usual "fish-tank" employed for paper chromatography.

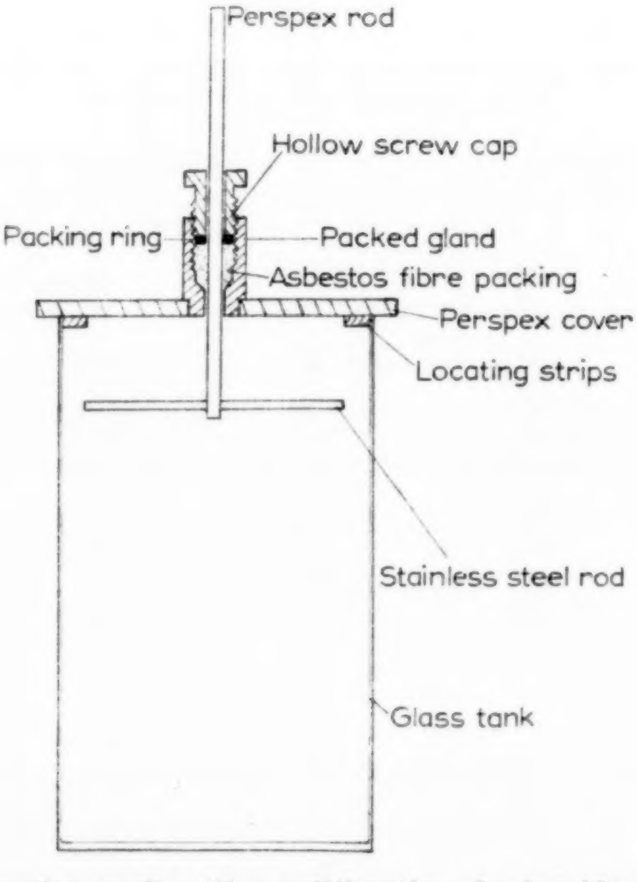

Fig. 1. Chromatography tank with equilibration device (drawing not to scale).

The device, shown in Fig. 1 (which is not drawn to scale), consists essentially of a vertical rod of circular cross section fitted through a packed gland in the middle of the cover, and having a horizontal bar attached centrally at the lower extremity. This bar carries clips (or, more simply, clothes pegs), from which the paper strips may be suspended for ascending chromatography. The gland is provided with an internal screw thread, and the packing can therefore be compressed or loosened by adjustment of a screw cap. By means of this arrangement, the bar carrying the papers may be raised or lowered without opening the tank; thus the papers may be suspended in the tank above the level of the solvent, and then lowered into the solvent after saturation with the vapour is considered to be achieved.

Fig. 2 illustrates the gland in greater detail. The hollow screw, A, presses on a plate B lying on asbestos fibre packing C, which seals the space between the vertical rod D and its enclosing gland. As long as the packing is loose, the rod D may be raised or lowered at will, but on tightening A the packing becomes compressed, thus locking the rod in position and sealing the gland.

The device described in this instance was made of "perspex" and for use in ascending chromatography. The same principle, of course, could be used for material more resistant to the action of solvents, and likewise it would not be difficult to adapt the method for descending chromatography.

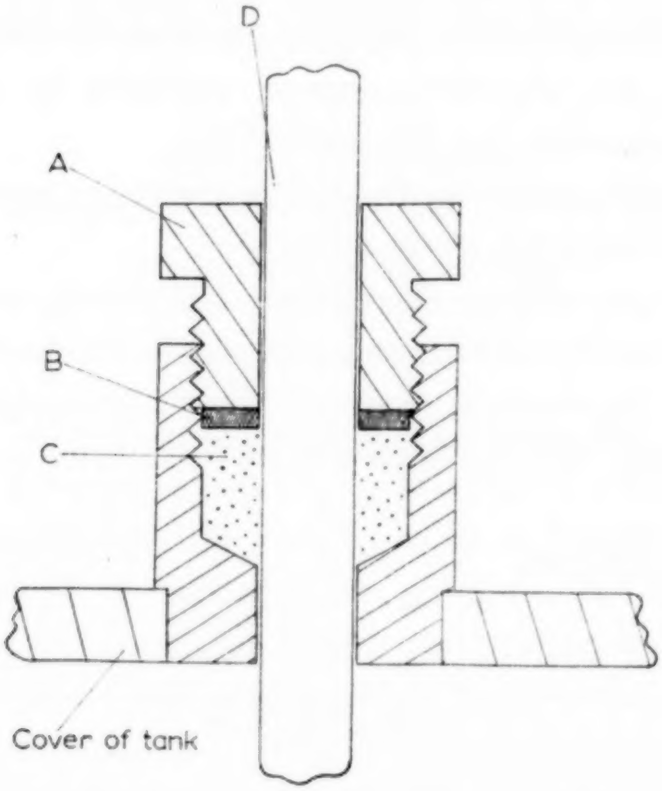

Fig. 2. The packed gland. A = hollow screw. B = packing ring. C = asbestos fibre packing. D = vertical rod. Scale is actual size.

*Department of Chemical Engineering, University College,*
*London (Great Britain)*

R. S. Potter
E. Margery Linday
R. Chayen

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## BOOK REVIEW

*Protides of the Biological Fluids*, Proceedings of the Sixth Colloquium, Bruges, 1958. Edited by Dr. H. Peeters, published by Elsevier Publ. Co., Amsterdam, 1959, 330 pages, price 45 s.

Following the publication of the reports of the Fifth Colloquium on Protides of the Biological Fluids (Bruges, 1957), all the communications of the Sixth Colloquium, held also at Bruges in 1958, have now appeared.

It should be pointed out that some of the contributions to this Colloquium have already been published in *Clinica Chimica Acta*, but the great advantage of publishing the complete Proceedings is that of collecting all this material in a single book, whose practical use and great interest in the field of medical applications for all biologists, chemists and analysts is self-evident.

This volume, like the previous one, has been edited by Dr. H. PEETERS. The papers have been grouped under the following headings: general topics, such as chromatography of proteins; techniques, such as micromethods of electrophoresis, etc.; special topics: macroglobulins, isolated protein fractions, protides and reproduction, protides and the digestive system, protides of the cerebrospinal fluid, protides in disease, therapeutic use of amino acids.

The subjects for the Round Table Discussions were: protein pattern in normal and pathological cases, and electrophoretic techniques.

This volume will assuredly be of great use to chemists, analysts and pathologists. It demonstrates the great revolution and progress in the methods and in the studies of protein systems by electrophoretical and chromatographic techniques, in a field in which, only a year ago, the conventional analytical methods had failed.

G. B. MARINI-BETTÒLO (Rome)

*J. Chromatog.*, 3 (1960) 203–204

## NEW BOOKS

*Die Papierelektrophorese,* by CH. WUNDERLY, 2nd Ed., H. R. Sauerländer & Co., Aarau and Frankfurt am Main, 1959, 202 pages, price Sw. Frs. 18.80.

*Studies in Crystal Physics,* by M. A. JASWON. Reprinted from *Research* (*London*), Vol. 11 (1958), Butterworths Scientific Publications, London, 1959, 42 pages, price 10 s. 6 d.

Five papers on crystal physics, each touching upon a different topic but all concerned with physical effects related primarily to the crystal structure rather than to the specific kind of atom involved, and providing the thermodynamic background to the picture.

*Gas Chromatography,* by A. I. M. KEULEMANS, edited by C. G. VERVER, 2nd Ed., Reinhold Publishing Corporation, New York, 1959, xxi + 234 pages, price $ 7.50.

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REVIEW

# MULTIPLE ZONES AND SPOTS IN CHROMATOGRAPHY

ROY A. KELLER
*Department of Chemistry, University of Arizona, Tucson, Ariz.*

AND

J. CALVIN GIDDINGS
*Department of Chemistry, University of Utah, Salt Lake City, Utah (U.S.A.)*

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The tendency among workers employing chromatography to accept two or more zones on a column or spots on a paper as indicative of heterogeneity has almost led to some erroneous conclusions. The careful work of Smith[1] and Ovenston[2] which detected the anomalies in their particular investigations responsible for what has become known as multiple or double zoning or spotting is deserving of praise. It is difficult to say how many "new compounds" have been reported because of this phenomenon. It is now recognized that a single pure substance may lead to the formation of more than one zone or spot[3]. Multi-zoning is particularly dangerous with mixed chromatograms where an unknown and a known compound are mixed and chromatographed. If two spots appear the compounds are assumed to be different which may not be the case at all. Ovenston[2] advises that the unknown and suspected known be chromatographed side by side so double spotting, if it occurs, will be obvious. As Ovenston remarks, theoretically a single substance should give a single zone but actual chromatographic systems may be far more complex than the assumed theoretical model. Explanations may be given for particular cases but no one seems to apply to all[4]. It is the purpose of this communication to review, summarize, and perhaps extend some of these explanations.

## SOURCES OF MULTI-ZONING

### *Chemical reaction*

Some solutes may, unknown to the investigator, undergo chemical conversion from one form, or species, to another prior to application to the chromatogram, after application but before development, or during the course of development. Cases of reaction on active adsorbents have been known for some time[5]. Alumina is notorious in this regard. This problem and some possible mechanisms for the action have been extensively discussed in an interesting manner by Meunier and Vinet[6]. If the rate of A going to B is finite but the reverse reaction is very slow, or does not occur at all, then in the course of development of A, there should be two contiguous zones, one of A and the other of B if the two compounds have different mobilities. The time interval where the two coexist becomes shorter the faster the reaction. Rechromatography should detect this situation. In the same system, B should give one zone while A should still give two. Eventually B should be the only compound detectable.

If the immobile phase is nonparticipative in the conversion, the reaction may be oxidative or photosensitive[7,8].

Reactions that occur before development is started lead to multiple spots. BAYLY, BOURNE AND STACEY[9] found that glucose chromatographed in the presence of some ammonium salts gave three distinct, well separated spots. They later reported[10] that one of these was due to glucosylamine and another was diglucosylamine which were formed when the solution of glucose containing the ammonium salts was applied to the paper and dried in a stream of warm air prior to development. These artifacts were not formed if the origin was dried in cool air. Since the reaction attained equilibrium before development, the spots were distinct. These experiments were repeated for mannose, glucose, and galactose by RAACKE-FELS[11] who proposed for mannose a series of reversible equilibria between mannose, mannose–ammonia, mannosylamine, and dimannosylamine. The reversible reactions of mannose–ammonia proceed at rates which do not permit resolution on the chromatogram. The author proposes that the reaction is between the sugar and ammonia rather than ammonium salts, the latter being catalysts. Also, heat is apparently not necessary.

### *Impurities in the solution to be chromatographed*

STRAIN[12] has pointed out that extracts from plants containing pigments many times contain colorless components which lead to odd results. The inexperienced researcher, intent upon the pigments, may inadvertently perform some manipulations which lead to some unusual performances of the materials and to false conclusions. These adsorbed, colorless impurities may act as displacers during development. A solution of 2 to 3 mg of lutein or zeaxanthin per 100 ml of petroleum ether–25 % acetone and containing a small amount of a strongly adsorbed material, *e.g.*, propylene glycol, when developed with the petroleum ether–acetone solvent on a magnesia column gave a dark leading zone followed by a second faint zone. The two zones appeared separated by a zone devoid of pigment. A weakly adsorbed contaminant can give a faint leading zone and a contiguous dark second zone. In both cases, prolonged development gives a single zone.

The addition of a contaminated pigment solution to a column followed by a second, uncontaminated solution of the same pigment in the same solvent may lead to two zones which later recombine on prolonged development. If the two pigment solutions are mixed before addition to the column, one zone is produced. This observation should be a warning to those who combine extracts on a column.

The appearance of a double zone is given when the initial zone contains several fold the amount of material that can be dissolved in the developer occupying this zone (overloading). The solute may be regarded as "precipitated" relative to the developer. It is dissolved at the tail of the initial dark zone, is moved through the band and appears as a faint zone, leading the initial zone and contiguous with it.

Band inversions may temporarily give the false impression of multiple zoning. If A is above B when a solution of the two in solvent 1 is placed on the column and if the order is reversed with adsorption from solution in solvent 2, then on formation of the chromatogram with solvent 1 and development with solvent 2, A will migrate through B and one zone appears to be present during the inversion. Prolonged development gives two zones.

### *Discontinuities in the immobile phase*

Columns of kieselguhr treated with a liquid are generally accepted as the clearest example of liquid–liquid partition chromatograms[13]. The immobile liquid phase seems to consist of micro-drops dispersed through the inert support, where it is loosely held and subject to change particularly with developers which are not exactly saturated with the immobile phase. SMITH[1] chromatographed the B vitamins on kieselguhr holding one-half its weight of water or dilute buffers suspended in water-saturated *n*-butanol and developed with water-saturated *n*-butanol. If a portion of *n*-butanol containing excess water dispersed as colloidal droplets is passed through the column, the kieselguhr removes the water to give a locally continuous wet phase at the top of the column which could trap a portion of the solute. If this is developed with water-saturated *n*-butanol, the untrapped solute is washed from this region to give a band lower down on the column and leaving a band at the top. Since the immobile phase no longer exists as small droplets, equilibrium could not be established with the mobile phase and the solute is only slowly leached from the upper portion. This leads to two bands joined by a very pale region. If the column is first treated with butanol containing less than the saturation amount of water, immobile phase may be removed from the top portion of the column to give a "dry" region which can lead to double zoning on development with water-saturated *n*-butanol. Since the solubility of water in butanol is strongly temperature-dependent, these same discontinuities can be produced by a temperature variation of as little as 5° during chromatography.

OVENSTON[2] had a similar experience with silica-"Celite" columns whose adsorptive properties also depend on water content. When the column was prewashed with diethyl ether, the ether partially eluted water from the column. When the ether wash was stopped before the excess water was completely removed and the chromatogram formed and developed with a different solvent of weaker eluting power, an intermediate wet zone was formed on the column which led to double zoning at the discontinuity. Petroleum ether will remove the ethyl ether without affecting the wet zone. The two solute zones, once passed the discontinuity, will eventually reunite in this case. However, if the developer also causes the wet zone to migrate at a rate comparable to the adsorbate, they may not reunite. OVENSTON warns that double zoning is likely to occur if the column is first treated with a strong developer either in a prewashing treatment or during chromatography and is followed by a weaker developer. It would seem preferable to prewash adsorbents by decantation before packing the column to avoid such discontinuities.

A similar explanation may apply to the double zoning observed by Schroeder[4] on silicic acid columns.

Large amounts of inorganic salts in the solution applied to the chromatogram may produce water-logging near the origin by a salting out effect[14].

### *Discontinuities in the mobile phase*

Boman[15] applied human serum albumin along an oblique line on a sheet of paper and developed the chromatogram with ethanol–phosphate buffer. The final spots should also have been on an oblique line. However, they appeared on a horizontal line. The explanation given is that the albumin travels close to the developer front and during its journey down the paper is partly adsorbed by the paper. It is soon all adsorbed to give a series of narrow elongated spots nearly parallel to the initial oblique line. The developer front appears to be followed by a "second front" which causes some change in the paper, possibly in electrical charge, which releases the albumin which then travels with this front. This front, parallel to the first, aligns the spots on a horizontal line. In certain cases, the solute appears as a double spot, one between the first and second front and one at the second front. No real discontinuity need exist in the mobile phase. If some sort of gradient of some property exists in the developer in the direction of flow and if at some particular value of this property the albumin is released, then the chromatogram gives the appearance of two fronts in the mobile phase. This sudden change of adsorbate may be pictured as similar to the sudden change in pH at the end point when a strong acid is slowly added to a strong base in the course of a titration. Nunes da Costa and Guedes de Carvalho[16] have observed double zoning accompanying double fronting in the paper chromatography of the borates.

### *Charged species and complexes*

Whenever a solute can exist as one or more uncharged species and/or as one or more charged species, or is capable of forming complexes, multi-zoning can occur. This is probably the least understood cause of multiple spots.

Landua, Fuerst and Awapara[17] found that tailing and double spotting of amino acids depended strongly on the pH of the solution applied to the paper. All compounds were developed with phenol–water. The observation that the solutes could exist as one or more species and that double spots were always connected by a diffuse region is important as will be shown later. Aronoff[18] found the same situation for lysine and that the relative intensities of the spots were in rough agreement with the distribution of ionic species as calculated from the pH of the solution applied. The lysine ions would be capable of association with the phenol to give new compounds with their own partition coefficients. Westall[19] has proposed a similar association to explain the separation of sodium and chloride ions on paper when developed with phenol–water. The sodium ions supposedly form a phenate which is more soluble in the phenol and shows a higher $R_F$.

Various dissociations may be visualized. If the solute is capable of accepting a proton

$$A + H^+ = HA^+$$

the ion formed may associate with phenate ion

$$HA^+ + C_6H_5O^- = AHOC_6H_5$$

Ionic species would be retained by the polar non-mobile aqueous phase while the unionized compound formed with the phenol would be more soluble in the mobile phase and migrate with a higher $R_F$. The initial distribution of charged and uncharged species would be fixed by the pH of the solution applied. If development disturbs the acid concentration, and it is difficult to see why it shouldn't since the pH of the developer is not adjusted by a buffer, the equilibria should readjust to that given by the solute in phenol–water and the chromatogram obtained should be the same in all cases, that is, independent of the initial pH. The fact that this is not true indicates a more complicated change. As will be shown, definite discrete double spots will be obtained only if the rate of conversion is slow compared to the time of development of the chromatogram. If the rate of conversion is fast compared to the time of development, one spot should be obtained. Between these two extremes, serious tailing and multiple spots joined by diffuse regions may occur. This argument, of course, makes the assumption that in the earlier stages of development, the hydrogen ion causing the initial distribution is removed from the origin and that the system is either irreversible or attempts to attain the distribution it would have in the developer. That the first part of this assumption is questionable is shown by WALDRON-EDWARD[14] mentioned below.

The rate of interconversion of charged and uncharged species is not the only factor, however. CURRY[20] observed multiple spots when he chromatographed the different acid phosphate salts in pyridine–ethyl acetate–water. The number and intensities of the spots depended upon the pH. If orthophosphoric acid is used, the faster spot predominates; if the normal salt of this acid is used, the slower spot predominates. He assumed that the spots were due to $H_2PO_4^-$, $HPO_4^{-2}$ and $PO_4^{-3}$ and that pyridine complexes were not involved since similar results were obtained with butanol. When orthophosphoric acid with radioactive phosphorous was used, the activity was 6000 counts/min at the spots but only 100 counts/min between spots showing the spots to be well resolved. This could be explained by a slow proton transfer

$$H_2PO_4^- = HPO_4^{-2} + H^+$$

To test this he chromatographed a mixture of labeled $H_3P^*O_4$ which ought to give $H_2P^*O_4^-$ and unlabeled $Na_2HPO_4$ which ought to give nonradioactive $HPO_4^{-2}$. Both final spots obtained were radioactive indicating an appreciable rate of proton transfer. A close examination of CURRY's paper indicates that the tagged and untagged species were mixed before application to the chromatogram. This would conceivably permit proton transfer before chromatography and one would expect activity in both spots. He did not feel that multispotting was due to the interference of inorganic ions or impurities in the paper since the paper was acid-washed which generally is the accepted procedure for removal of such impurities. Multispotting was observed with 2′,3′-isopropylidene-adenosine-5′-phosphate. ERDEM'S[21] chromatograms with arsenates were very similar to CURRY'S. ERDEM also obtained two to three distinct spots with divalent cadmium in the presence of ammonia. All of the spots contained cadmium and were attributed to different amino complexes. ERDEM AND ERLENMEYER[22] point out that one must assume that the kinetics of the transformation are radically different in the immobile phase than in the mobile phase. They cite examples from the literature of adsorption as justification. Their assumption requires that paper chromatography involves an adsorption mechanism rather than a partition between an immobile liquid-gel and a mobile fluid as proposed by MARTIN[23] and discussed by MOORE AND STEIN[13]. MARTIN[24] suggests that reaction rates in the liquid-gel do not differ much if at all from those observed in the pure liquid. The suggestion of radically different reaction rates in the immobile phase in paper chromatography must be regarded with suspicion unless more supporting evidence is found.

Impurities in the paper may very well cause unusual results. HANES AND ISHERWOOD[25] working with inorganic phosphate, traced "ghost spots", or retention of some of the solute at the point of application, to the presence of calcium and magnesium ions in the paper. This was eliminated by prewashing the paper with acid. These ions are also responsible for "shadows", faint regions following the principle spot but different from tailing. Multiple banded spots, which give the appearance of a well defined dark spot overlapping a fainter but equally well defined second spot, could be overcome by a strong organic acid in the developer.

WALDRON-EDWARD[14] found that D-glucosamine gave two spots when sulfate ion was present in the solution applied to the chromatogram in an amount equivalent to the amine; that the slower spot only was produced with excess sulfate and that the second spot always contained sulfate which was not present anywhere else in the developed paper. Similar effects were found with lysine, arginine, ornithine, histidine, ethanolamine, tyramine, ethylene diamine, and tetramethylene diamine. Some of these same substances were found to give unusual behavior by LANDUA, FUERST AND AWAPARA[17] and by MCFARREN[26] who investigated amino acid chromatography on buffered papers.

Earlier it was assumed that the developer separated the solutes from the inorganic ions of the buffer in the solution applied to the chromatogram thus disturbing the equilibrium. If these ions migrate with the solutes, however, they may maintain the equilibrium or the position of equilibrium may be changed only slowly by their gradual removal. Such a possibility increases the complexity of the problem. Not only are rates of conversion important but also the position of the equilibrium. The latter may be fixed by a buffered paper which may partially account for the success of this technique.

The problem of inorganic salts and their role in salting out substances, forming complexes, and migrating with developer is a formidable one. If one adds to this their possible participation in an ion exchange mechanism[27] the situation becomesworse.

### *Equilibrium between two species*

Possibly one of the best examples of reaction between two forms of the same solute and which is free of disturbing influences such as inorganic ions is given by PARTRIDGE AND WESTALL[28] who chromatographed the sugars and related compounds on paper using phenol, *s*-collidine, and *n*-butanol–acetic acid. Neutral reducing sugars gave well defined spots indicating that the tautomeric equilibria known to occur in water did not affect the chromatogram. In butanol–acetic acid a mixture of glucuronic acid and its lactone, glucurone, gave two well separated spots; glucuronic acid gave two well defined and unjoined spots while glucurone gave a very distinct lower spot and a smaller, fainter, well separated slow spot. In *s*-collidine, glucuronic acid gave a dark slow spot and a smaller, fainter faster spot; glucurone gave two spots joined by material all the way along the chromatogram. In general, the leading spot showed tailing while the slow spot showed bearding. This observation is in accord with theory. If a substance exists in two forms which are interconverting and if the rate of conversion is slow relative to the time of development, then two spots can be obtained. There would be material joining these spots all the way along the chromatogram but its detection depends on the sensitivity of the method of revelation. The lead zone should show tailing while the slow zone should show bearding.

Theoretical treatments of differential migration have been given for sedimentation and electrophoresis[29–31]. If one accepts that these processes all represent the same type of phenomena, that is, differential migration of substances in a phase when subjected to some driving force, then these results should be qualitatively adaptable to chromatography. We will outline, in detail, a method for calculating concentration profiles for general differential migration methods and a method for calculating spot shapes in paper chromatograms.

## INTERCONVERTING SPECIES

It is a little known fact that a large class of chromatographic anomalies can appear as a result of an involved kinetics. It is a general requirement that one of the kinetic steps be slow so that the system is not near equilibrium. The kinetic scheme discussed below, which is just one example of many possible schemes, leads to the formation of tails, comets, beards, double spots, and even triple spots, with the appropriate choice of parameters.

Suppose that a substance can exist in two different, interconvertible forms, A and B, each with a degree of permanence shown by a slow rate of conversion between them. Suppose, also, that each form undergoes the usual chromatographic exchange (adsorption or partition) between the mobile and immobile phases and back again. This exchange is denoted by $A \rightleftharpoons A'$ where A designates a molecule of the species A in the mobile phase and A′ designates a molecule of this species in the immobile phase. A similar expression can be written for species B, *i.e.*, $B \rightleftharpoons B'$. Both of these exchanges are assumed to proceed rapidly. The net scheme is

$$\begin{tikzcd}[column sep=large]
& \text{Slow reaction} & \\
A \arrow[d, shift left] \arrow[rr, shift left, "k_1"] & & B \arrow[ll, shift left, "k_2"] \arrow[d, shift left] \\
A' \arrow[u, shift left, "\text{Fast reaction}"] & & B' \arrow[u, shift left, "\text{Fast reaction}"']
\end{tikzcd}$$

The slow reaction between A and B proceeds with first order rate constants, $k_1$ and $k_2$.

*Zero rate constants*

The investigation of the above scheme can be best approached by first assuming $k_1 = k_2 = 0$, *i.e.*, with no interconversion between A and B. Each species will migrate independently of the other and the zone of each will broaden during migration. This broadening is due to the combined result of the partitioning kinetics, molecular diffusion, and "eddy" diffusion[32, 33]. Each of these phenomena can be assigned an apparent diffusion coefficient to describe its contribution and the sum of these gives an effective diffusion coefficient $D$ which describes the spreading of the zone. When $D$ itself is variable (due to non-constant flow rates, etc.) then a time average of $D$ must be used. If the initial zones are very narrow bands and a diffusion model describes the spreading, then the concentration profiles, at any later time during development are Gaussian in shape.

The picture then, with zero rate constants, is of two zones migrating with their own $R_F$'s and each spreading according to their individual values of the net diffusion coefficient $D$. If the mobilities of A and B are sufficiently different and zone broadening is not too great, well defined and separated spots should result. This case corresponds to the observations of BAYLY, BOURNE AND STACEY[9], and RAACKE-FELS[11].

This picture is modified for the reversible conversion of A to B with finite transition rates. The following simple argument shows the nature of the new concentration profiles. Any molecule that was originally A and has changed to B or *vice versa*, has spent part of its time migrating with an $R_F$ characteristic of A, and the remainder of its time with the $R_F$ of B. Hence at a later time, $t$, such a molecule will be found somewhere between the A and B zones at the two extremes. Thus there will appear two zones or spots with a diffuse streak between them.

The question next arises as to the amount of material in the two extreme zones relative to the material between them. Since the reactions are all first order, the end zones disappear by exponential decay. If the original material is divided with the fractions $\alpha$ and $\beta$ in the A and B forms respectively, then at the later time $t$, $\alpha \exp(-k_1 t)$ will be the fraction in the A form and $\beta \exp(-k_2 t)$ will be the fraction in the B form. The total fraction of material in the intermediate streak will be $1 - \alpha \exp(-k_1 t) - \beta \exp(-k_2 t)$. At sufficiently long times, compared to both $1/k_1$ and $1/k_2$, this will approach unity and essentially all the molecules will be somewhere between the expected positions for A and B with no interconversion. Under the condition of large $t$, nearly every molecule will have made several $A \rightleftharpoons B$ transitions, and the concentration profile will approach a Gaussian distribution with the peak somewhere in the center, its position depending on the relative average times spent in the A and B forms.

A quantitative evaluation of the concentration profile can be made using chromatographic theory[33a]. In its simplest form, this theory gives the probability distribution for the relative times spent in the A and B forms when the reaction scheme is, as above, $A \rightleftharpoons B$. If we let the fraction of time that a molecule spends as A be $x$, then the probability that this fraction is in the range $x$ to $x + \mathrm{d}x$ is

$$P_1(x)\,\mathrm{d}x = a \exp\left[-a\,(1 - x) - bx\right] I_0 \sqrt{4abx\,(1 - x)}\,\mathrm{d}x \tag{1}$$

providing that the molecule was in the A form at the beginning of the run and in the B form at the end. In this expression, $a = k_1 t$ and $b = k_2 t$. $I_0$ is a Bessel function of imaginary argument.

If the molecule starts out as A and after at least one reaction cycle, ends as A, then the probability becomes

$$P_2(x)\,\mathrm{d}x = \left[\frac{ab\,(1 - x)}{x}\right]^{1/2} \exp\left[-a\,(1 - x) - bx\right] I_1 \sqrt{4abx\,(1 - x)}\,\mathrm{d}x \tag{2}$$

The only other possibility, starting with A, is that no reaction at all occurs. The probability of this case, for which $x = 0$, is $\exp(-a)$.

The remainder of the concentration profile comes from the molecules that are originally in the B form. The $P(x)$ expressions in this case are obtained from the previous equations by substituting $a$ for $b$ and $(1 - x)$ for $x$. The corresponding equations, first for a molecule that begins as B and ends as A (eqn. (3)), and second for a molecule that begins as B and returns to B after one or more reaction cycles (eqn. (4)) are

$$P_3(x)\,\mathrm{d}x = b \exp\left[-a\,(1 - x) - bx\right] I_0 \sqrt{4abx\,(1 - x)}\,\mathrm{d}x \tag{3}$$

$$P_4(x)\,\mathrm{d}x = \left(\frac{abx}{1 - x}\right)^{1/2} \exp\left[-a\,(1 - x) - bx\right] I_1 \sqrt{4abx\,(1 - x)}\,\mathrm{d}x \tag{4}$$

The probability that no reaction occurs, for which $x = 1$, is $\exp(-b)$.

The overall concentration profile is proportional to the final probability density function, $P(x)$, which is obtained by properly weighing the above expressions. Since the original fractions of molecules in the A form is $\alpha$ and in the B forms is $\beta$, then

$$P(x) = \alpha\,[P_1(x) + P_2(x)] + \beta\,[P_3(x) + P_4(x)] \tag{5a}$$

$$P(x = 0) = \alpha \exp(-a) \tag{5b}$$

$$P(x = 1) = \beta \exp(-b) \tag{5c}$$

We have computed $P(x)$ for several different sets of the parameters $a$, $b$, $\alpha$, and $\beta$. In the typical case, $P(x)$ is continuous and finite in the interval $0 < P(x) < 1$. At $x = 0$ and $x = 1$, an infinitely thin slice has an area on the normalized $P(x)$ curve of $\alpha \exp(-a)$ and $\beta \exp(-b)$. Since this cannot be shown graphically, we have included this area in a Gaussian distribution centered about $x = 0$ and $x = 1$ and with a finite standard deviation of $\sigma = 0.1$. This procedure, for a typical case, is illustrated in Fig. 1.

The distribution of material shown by the $P(x)$ curves in these figures results only from the A $\rightleftharpoons$ B reaction and has not yet included the actual chromatographic process A $\rightleftharpoons$ A′ and B $\rightleftharpoons$ B′. This situation is easily remedied, however, since a single diffusion coefficient may be used to describe the combined effects of the chromatographic process, ordinary molecular diffusion, and eddy diffusion[32-34]. In order to calculate this, we set up a numerical program which allows for the diffusion of the material in the zone described by $P(x)$. The Schmidt method has been used for this purpose[35]. The calculations of this method can be made in less than an hour with the use of a desk calculator. We have restricted our considerations to the case where the diffusion coefficients of species A and B are the same. If it seems desirable to pursue the less restricted case where they are different, this can be easily done.

The choice of the diffusion parameters is independent of those chosen to represent the kinetics of the reversible conversion of A to B. We have kept ordinary diffusion at a minimum to emphasize the kinetic effects. In practical cases diffusion may often be a more important factor which would manifest itself simply as an additional spreading of the profile over and above that presented here. Our calculations are made with an effective diffusion coefficient

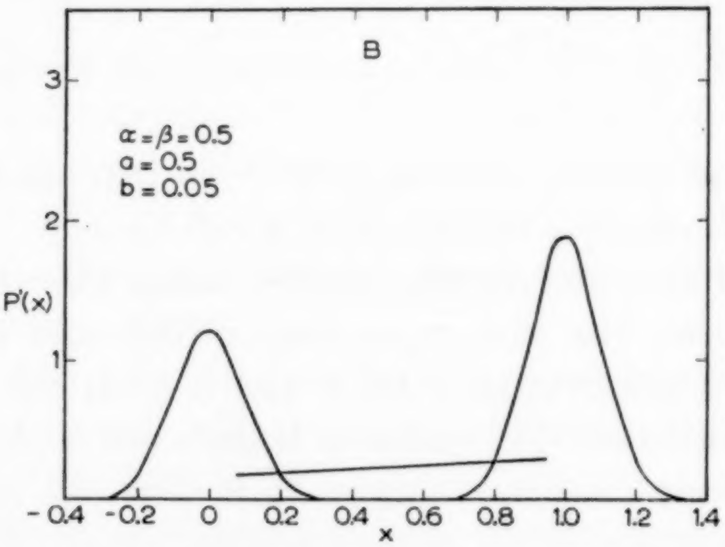

Fig. 1. The probability (concentration) density, $P(x)$.

$$D = \frac{[0.1\ (R_{FB} - R_{FA})\ v]^2}{2t} \tag{6}$$

where $R_{FA}$ and $R_{FB}$ are the $R_F$ values of the two species and $v$ is the velocity of the mobile or developer phase. This value was chosen so that the root-mean-square displacement of a molecule by diffusion is one-tenth the distance between the centers of the A and B zones. The new probability density function, $P'(x)$, is obtained by adding the "diffused" $P(x)$ profiles to the Gaussian curves representing the end zones. Fig. 6 is the final result.

## CALCULATION OF SPOT SHAPES

The $P'(x)$ profiles calculated above yield some representative concentration profiles which are useful if concentrations are measured directly as is often the case in the elution techniques. Quite often, however, zones or spots are observed visually,

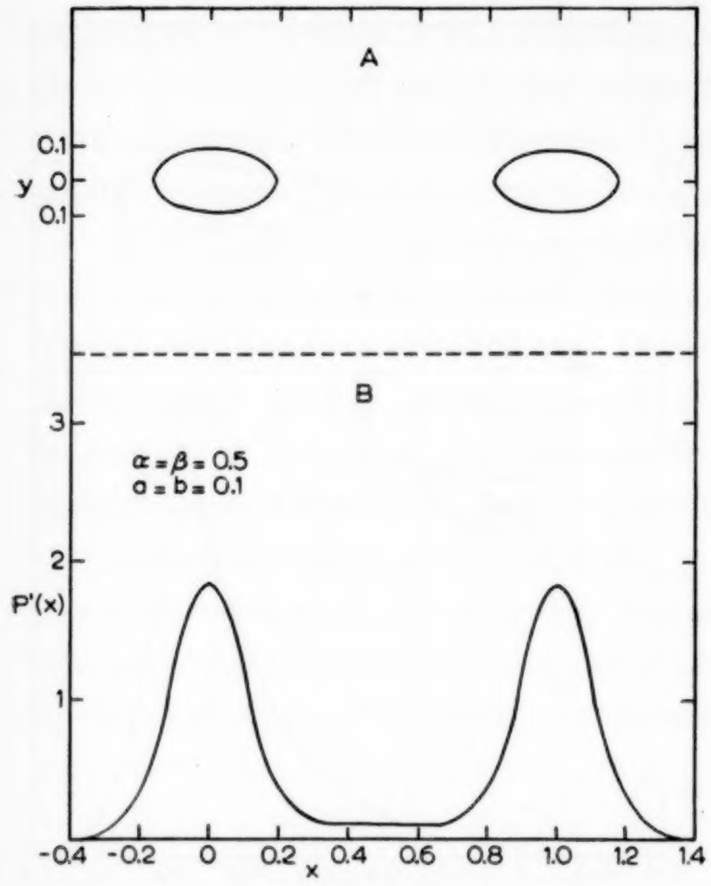

Fig. 2. Concentration profile and spot outlines for a symmetrical case. With a more sensitive method of detection, a continuous streak would exist between the two spots corresponding to the finite concentration between them.

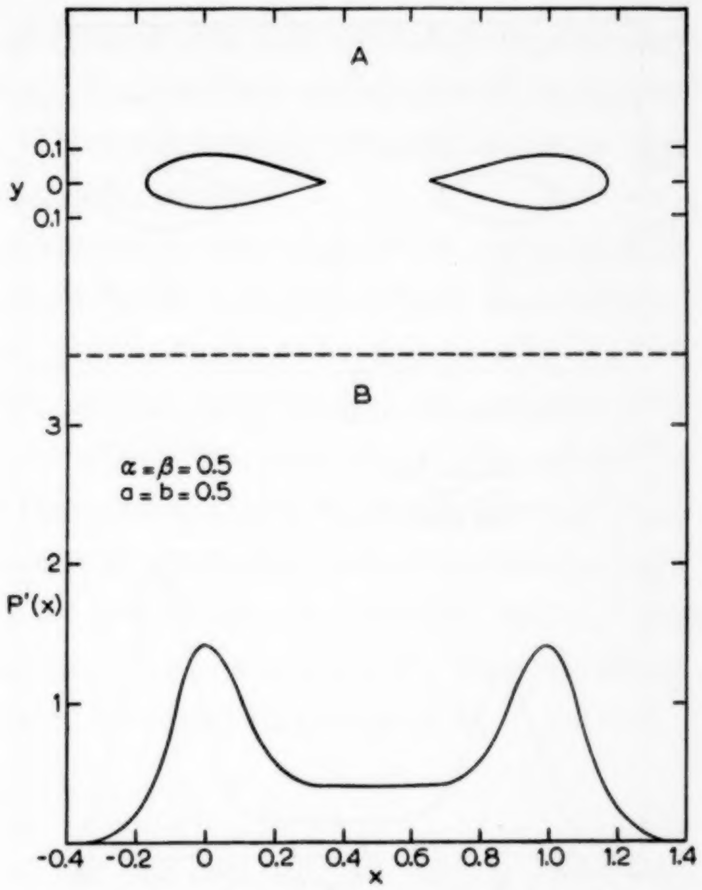

Fig. 3. A symmetrical case in which the two spots tail towards one another due to the increased concentration in the center.

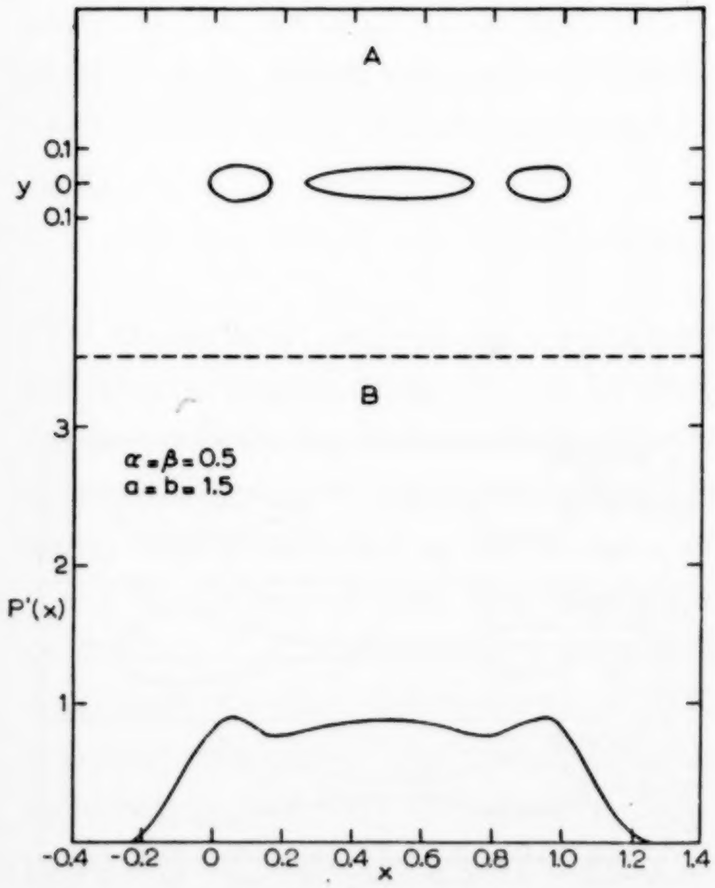

Fig. 4. Triple spots will be seen for this case if the initial spot content and the sensitivity (cutoff) fall within narrow limits. Since the minima in the concentration profile are not prominent, a streak would ordinarily appear between each spot. Otherwise the spots will be quite faint.

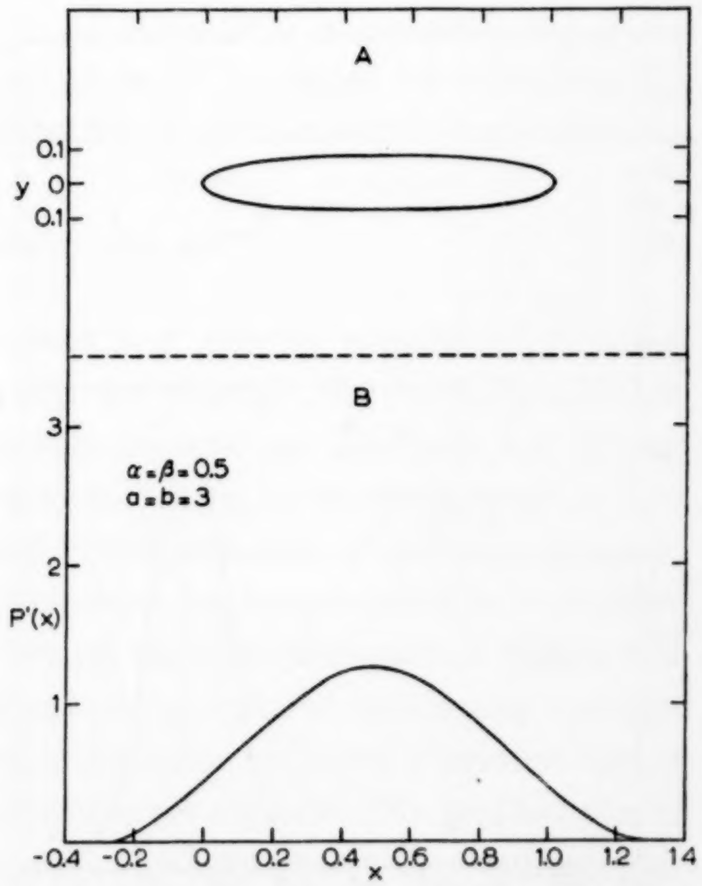

Fig. 5. Elongated spot resulting from the extended development of double spots with an origin in slow kinetic steps.

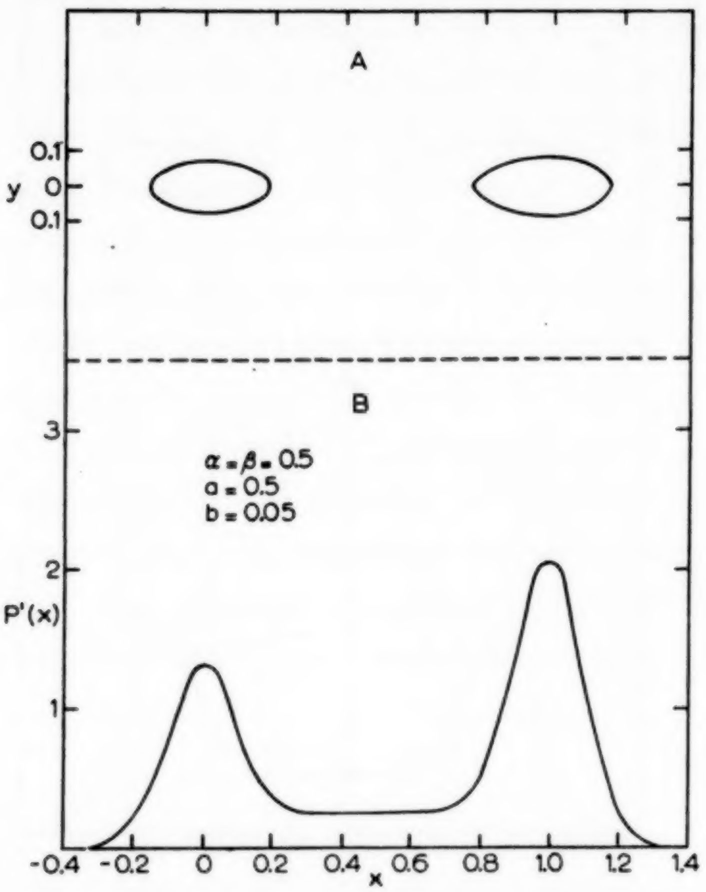

Fig. 6. Asymmetrical case. With a different spot content or cutoff this might appear as a single spot with bulging ends, double spots, or a single spot.

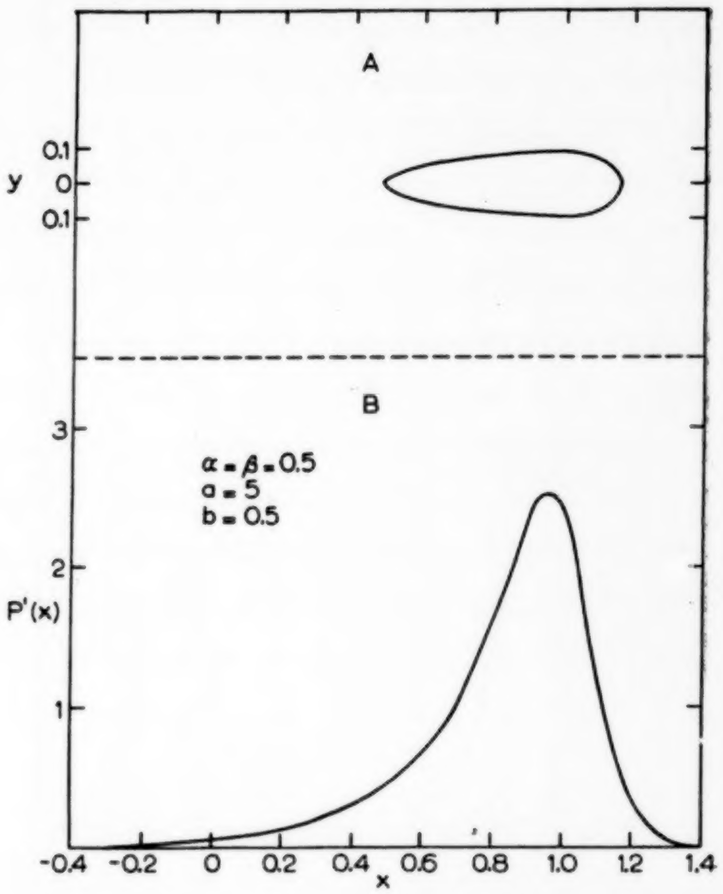

Fig. 7. Asymmetrical case in which one spot has essentially disappeared. Notice the extended tailing in the concentration profile and in the spot.

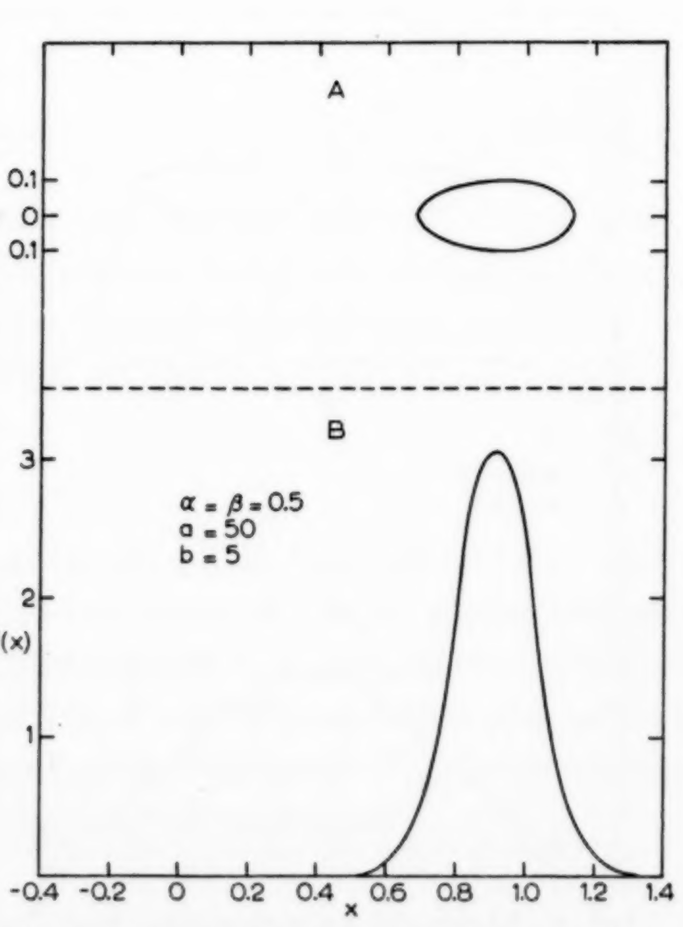

Fig. 8. Asymmetrical case with extended development. The spot appears elliptical.

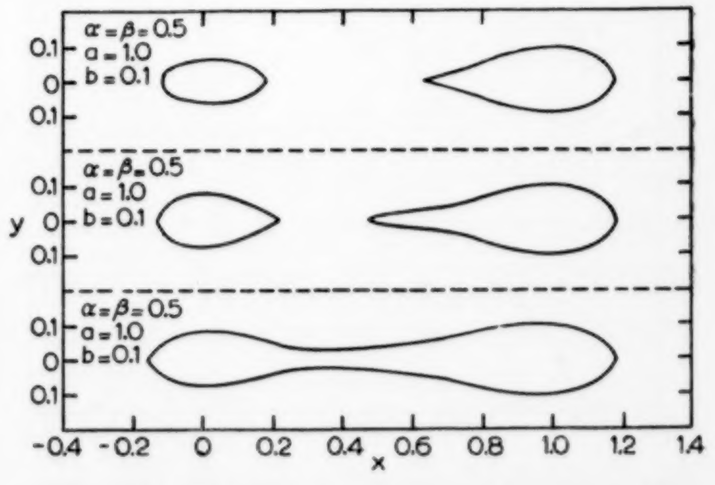

Fig. 9. Spot outlines at different cutoff values for an asymmetrical case. The range of phenomena that can be found is partially illustrated here. All the spots illustrated will, in practice, have a diffuse rather than a sharp boundary.

sometimes with the aid of a revelation reagent. In this case it is useful to express the above theory in terms of spot sizes and shapes which are encountered in paper chromatography. In this way the researcher is provided with suggestions as to the kinetic processes contributing to his chromatographic results.

In accordance with the previous communication in this series[33] we assume that the material spreads both in the direction of developer flow (longitudinal) and perpendicular (lateral) to it with different effective diffusion coefficients for the two directions. If the width of the original spot is small the lateral distribution of material will appear as a Gaussian distribution with $\sigma_y = (2D_y t)^{1/2}$ where $D_y$ is the effective diffusion coefficient in the lateral direction. On this planar chromatogram any material is apparent as a spot (*i.e.*, within the spot boundary) when the concentration exceeds a certain critical or cutoff value (determined by the limits of visibility, etc.). This value is obtained when the two-dimensional density function equals $P_0$. The following equation describes the lateral extension $y - \bar{y}$, of the spot in terms of $P_0$

$$P_0 = \frac{P'(x)}{\sqrt{2\pi}\sigma_y} \exp\left[-\frac{(y-\bar{y})^2}{2\sigma_y{}^2}\right] \tag{7}$$

The width of the spot is $w = 2\,(y - \bar{y})$ and this becomes

$$w = \sigma_y \left[8 \ln \frac{P'(x)}{\sqrt{2\pi}P_0\sigma_y}\right]^{1/2} \tag{8}$$

When $w$ becomes an imaginary number this is to be taken as zero. Spots with widths obeying this formula have been drawn in Fig. 2 through 9 along with the $P'(x)$ profiles from which they are obtained. The cutoff point is altered by changing $P_0$. It should be noted that the spot might appear thicker or thinner simply by variations in $\sigma_y$.

## DISCUSSION OF THE RESULTS

The theoretical analysis of concentration profiles is a general method of treating differential migration processes. In electrophoresis, for example, the same formulation applies simply by substituting the two velocities $v_A$ and $v_B$ for $R_{FA}v$ and $R_{BF}v$. Hence a substance composed of two slowly interconverting forms can appear as two zones and sometimes as three zones, in either electrophoresis or chromatography.

The problem of zone distribution in electrophoresis has been treated by a number of authors. CANN, KIRKWOOD AND BROWN[31] set up the one-dimensional differential equations for the distribution of two interconverting species undergoing electrophoretic migration. They were able to obtain a solution only for a limited set of parameters, but they did observe double zoning in several cases. This method seems more difficult than the present one for this type of calculation since they indicate the need of a computer to extend the range of their parameters.

The cases of zone and spot distribution that we have examined are necessarily limited. There are so many physical parameters entering the calculation that it is impossible to explore the entire range of them in a single writing. The group we have examined is divided into two categories. In Figs. 2 through 5 we have treated the symmetrical cases where $a = b$ and $\alpha = \beta$. Asymmetrical cases are treated in Figs. 6 through 9 where $a \neq b$ and $\alpha = \beta$.

In the symmetrical cases we have shown examples where $a$ and $b$ successively increase. This roughly represents the calculation of a given chromatogram at successively longer time intervals. Since our profiles are plotted against the reduced variable, $x$, the end zones do not move farther apart with increasing time as they do in practice. It should be noticed that the principle result of increasing $a$ and $b$ is that of reducing the end zones and filling in the region between them. This has proceeded far enough when $a = b = 1.5$ that there appear three peaks, the center one being comparable in size to the end ones. Circumstances favorable to the appearance of three peaks are not often found since the peaks, when they appear simultaneously, are never very prominent.Three peaks would be more commonly observed with three slowly reacting species.

For larger values of $a$ and $b$ the end zones rapidly become insignificant. Thus where $a = b = 3$ they are swamped by the larger amount of material in the center. The corresponding zone is not Gaussian, however, until $a$ and $b$ become larger.

The parameters for the asymmetrical cases have been chosen arbitrarily such that $a = 10\,b$. Thus while an equal amount of material starts in each zone, the A zone is depleted more rapidly. The results show an asymmetry that is very similar to cases where $\alpha \neq \beta$. Whether the asymmetry is caused by an inequality in initial distribution ($\alpha \neq \beta$) or by an inequality in rates ($a \neq b$), or both, can be determined by further development of the chromatogram. The further depletion of material from the end zones depends directly on $a$ and $b$, and if these are equal, the fractional depletion in a given time is always the same.

When $a$ and $b$ are unequal, as for the cases calculated, the relative depletions are unequal. Thus with $a = 10\,b$, the A zone disappears more rapidly than the B zone. This is seen in the successive Figs. 6 through 8.

With each of the Figs. 2 through 8, we have shown at least one spot outline. The encircling line represents the limit of detection around the edge of the spot. Since this limit varies with the method of detection, we have shown some examples where the concentration profile is the same but the spots differ because this limit, or cutoff, has been changed, or because the spot content is different.

With symmetrical parameters the spots are distributed equally on each side of the point $x = 0.5$. While in practice such a symmetrical distribution is not to be often expected, the calculations provide some useful prototypes. In the succession of examples we see two separate elliptical spots, two spots that are elongated towards their common center (with a different cutoff, this would become a thin streak between them), a case where triple spots are to be expected, and finally a single spot, nearly elliptical, indicating the accumulation of most of the material in the center region. This series illustrates the general tendency of two zones to merge into one on prolonged development.

The series of spots obtained with asymmetrical parameters show a similar trend.

It is noticeable that the smaller spot is more nearly elliptical while the larger spot tends to trail off in the direction of the smaller one. In the last two figures the material is nearly depleted from the left hand side and no spot appears there. Notice in Fig. 7 the definite appearance of tailing in the spot. Tailing can appear under a variety of circumstances, even when the isotherm and kinetics are linear.

The ideal spots shown here are not always closely reproduced experimentally. A number of spot imperfections, such as missing corners and bulges, can be attributed to the heterogeneity of the paper. The heterogeneous nature of flow in paper often displays itself in the uneven shape of the solvent front.

While we have computed a representative sample of spot shapes in chromatography, the meaning of the results should not be misconstrued. We have used sets of physical parameters that are certainly not unique in providing the resultant spots. An observed set of spots may have its origin in:

1. The theory outlined here with the physical constants used here to obtain a similar set.
2. The theory used here but with a different set of constants.
3. An entirely different theoretical basis as outlined in the earlier parts of this paper. In order to decide between the three possibilities a careful study of the system must, in all cases, be made. The attempt here, has been simply to show that an entire group of phenomena will often appear as a result of the intrinsic kinetics of the system.

## TABLE OF SYMBOLS

| | |
|---|---|
| $k_1$ | rate constant for A → B interconversion |
| $k_2$ | rate constant for B → A interconversion |
| $D$ | effective coefficient of longitudinal diffusion |
| $\alpha$ | fraction of material originally in the A form |
| $\beta$ | fraction of material originally in the B form |
| $t$ | time |
| $x$ | fraction of time that a molecule spends in A form |
| $a$ | $k_1t$ |
| $b$ | $k_2t$ |
| $P_1(x), P_2(x), P_3(x), P_4(x)$ | probability densities given in equations (1–4) |
| $I_0, I_1$ | Bessel functions |
| $P(x)$ | overall probability density before diffusion |
| $P'(x)$ | overall probability density after diffusion |
| $P_0$ | cutoff probability density in two dimensions |
| $\sigma, \sigma_y$ | standard deviation due to diffusion |
| $D_y$ | effective coefficient of lateral diffusions |
| $P_y$ | lateral coordinate |
| $w$ | width of spot |
| $v_A, v_B$ | velocities of A and B |
| $R_{FA}, R_{FB}$ | $R_F$ values for A and B |

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## SUMMARY

Cases in which multiple spots appear in the chromatography of single substances are given. The various theoretical interpretations of multiple spotting are discussed and related to one another. Theoretical equations are derived for the evaluation of concentration profiles and spot shapes when the origin of multiple spotting is the slow interconversion of chemical species. A number of examples which give typical spot profiles are shown. Methods for determining the actual origin of multiple spotting are discussed.

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# THE CHROMATOGRAPHY OF NUCLEOTIDES, NUCLEOSIDES, AND PYRIMIDINES AND PURINES ON ACTIVATED CHARCOAL

R. L. STAMBAUGH AND D. WRIGHT WILSON

*Department of Biochemistry, School of Medicine, University of Pennsylvania, Philadelphia, Pa. (U.S.A.)*

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## INTRODUCTION

It was found in certain experiments with radioisotope tracers in this laboratory that a better purification of uracil from radioactive contaminants could be achieved by a gradient elution of the uracil from charcoal. Further experiments demonstrated that this type of elution was applicable to other compounds of this type. Activated charcoal has been used as an adsorbent for pyrimidines and purines by numerous investigators[1-4]. However, no attempt was made to elute the adsorbed compounds selectively from the charcoal in those experiments. This paper describes a method for eluting the pyrimidines, purines, and their nucleosides and nucleotides selectively by a gradient elution procedure. The method is presented to show the feasibility of the chromatography of these compounds on activated charcoal. By judicious variation of the solvents, one may conceivably obtain better resolution of the adsorbed compounds.

## MATERIALS

### 1. *Charcoal*

40 × 60 mesh Darco charcoal was used as the adsorbent in all of the experiments. This was made by grinding and sifting a larger particle size (20 × 40 mesh) Darco charcoal, and collecting only that fraction which goes through a 40 mesh sieve, but not through a 60 mesh sieve. This 40 × 60 mesh charcoal is then sedimented repeatedly in distilled water, each time sucking off the fine material, which fails to settle rapidly, with a water aspirator. This removes the fine charcoal particles which invaribly clog the column if not removed. The larger particles, which sediment rapidly, are allowed to stand 48 h in conc. aqueous $HN_3$–ethanol–$H_2O$ (5:1:13, v/v/v) with one change of solvent. The charcoal is then washed repeatedly with distilled water until the water wash is neutral to litmus. The charcoal is stored in distilled water until ready for use. It should be mentioned that this charcoal is brittle, and care should be taken to avoid crushing the particles in this procedure.

### 2. *Solvents*

Gradient elution of the adsorbed compounds was carried out with aqueous $NH_3$, ethanol, *n*-propanol, and *n*-butanol. Reagent grades of concentrated ammonia and ethanol were found to have a sufficiently low optical density in the range 240 mμ to 340 mμ without further purification. However, reagent grades of *n*-propanol and *n*-butanol were found to contain considerable amounts of aldehyde impurities, which absorb light in the wavelength range 240 mμ to 340 mμ. It was found that these aldehydes can be reduced to alcohols by treatment with sodium borohydride. The reduction is carried out by letting 400 ml of the alcohol and 6 g of sodium borohydride stand for 72 h in a cool place. During this time the mixture is swirled occasionally. Finally, the alcohol is distilled under vacuum in a flash evaporator at 40°. The results of such a treatment are shown in Table I.

TABLE I

OPTICAL DENSITY OF *n*-PROPANOL AND *n*-BUTANOL COMPARED WITH WATER

| *Wavelength (mμ)* | *Optical density of* | | | |
|---|---|---|---|---|
| | *Reagent n-propanol* | *Reagent n-propanol treated with* $NaBH_4$ | *Reagent n-butanol* | *Reagent n-butanol treated with* $NaBH_4$ |
| 220 | 1.58 | 0.481 | ∞ | 1.92 |
| 230 | 1.11 | 0.075 | ∞ | 0.750 |
| 240 | 0.542 | 0.024 | ∞ | 0.264 |
| 250 | 0.376 | 0.021 | 1.73 | 0.065 |
| 260 | 0.528 | 0.030 | 0.890 | 0.039 |
| 270 | 0.668 | 0.023 | 0.482 | 0.023 |
| 280 | 0.672 | 0.004 | 0.218 | 0.001 |
| 290 | 0.590 | 0.002 | 0.102 | 0.000 |
| 300 | 0.348 | 0.000 | 0.057 | 0.000 |
| 320 | 0.043 | 0.000 | 0.025 | 0.000 |

It was found that these two alcohols were sensitive to oxidation on standing. Therefore, it is necessary to flush them free of oxygen by bubbling nitrogen gas through the alcohol, and to store them in brown bottles. When stored in this way, the alcohols retain their low optical density for several months.

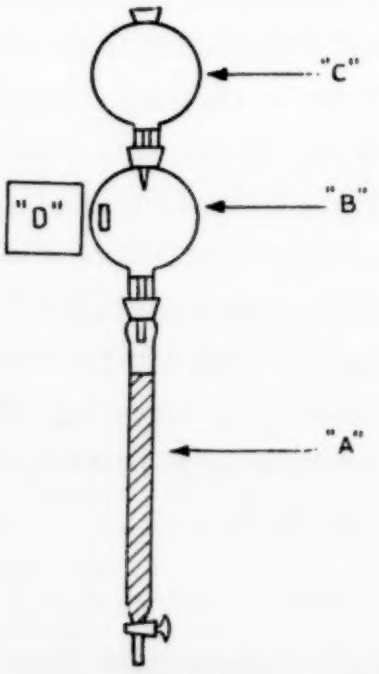

Fig. 1. Apparatus used for charcoal chromatography.

### 3. *Apparatus*

This consisted of a chromatography tube "A" and two 400 ml flasks, "B" and "C", all connected with ground glass joints as shown in Fig. 1. Flask "B" was stirred continuously with a magnetic stirrer "D". The solvent from flask "C" flowed through a fine capillary tip into flask "B".

## PROCEDURE

The compounds were adsorbed on a 1.5 × 15 cm column of Darco charcoal (40 × 60 mesh) in tube "A" by slowly running (0.25 ml/min) an acidic solution (pH 2.0) of the compounds through the column. The column was then washed with distilled water until the water wash was neutral to litmus. The elution of the adsorbed compounds was carried out as follows.

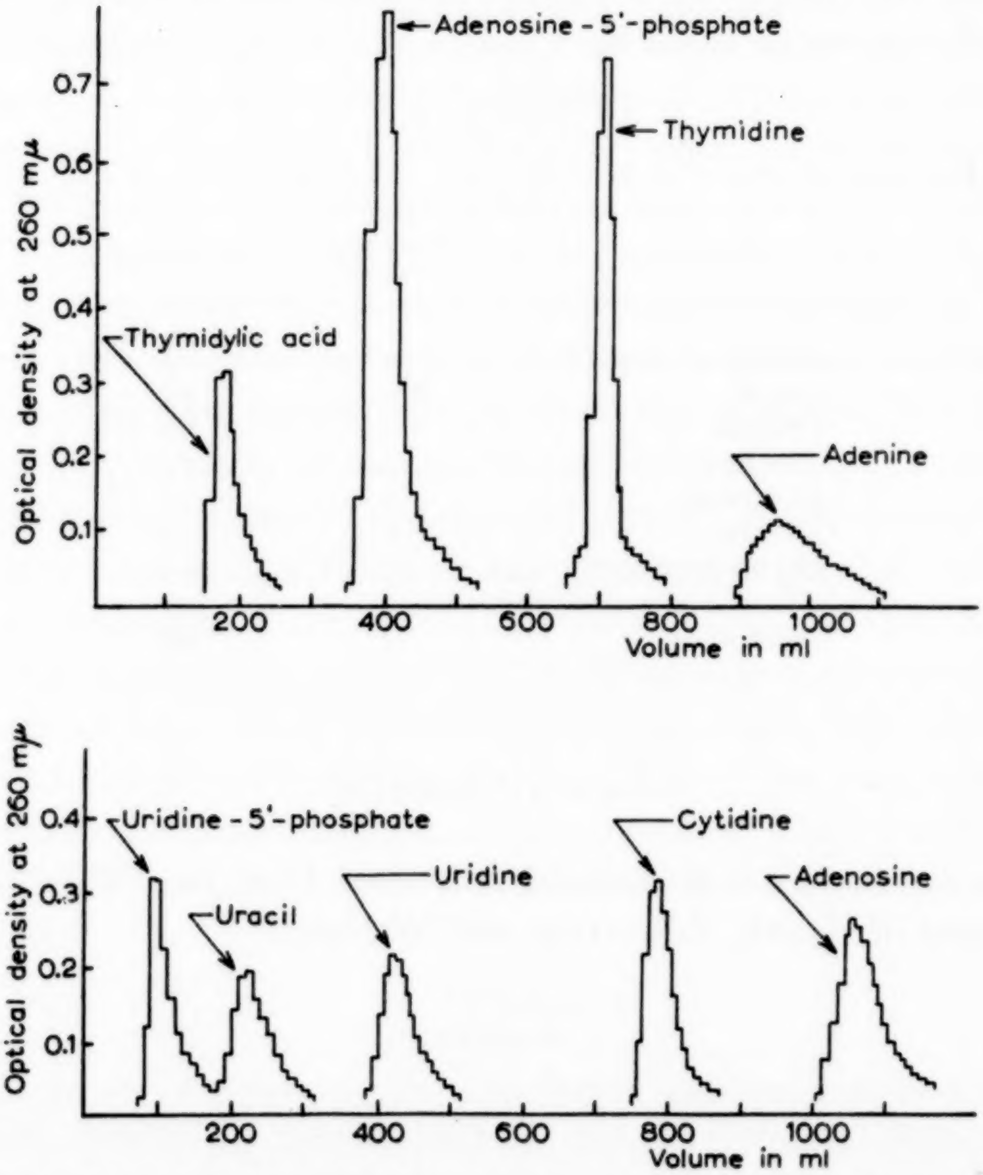

Fig. 2. Gradient elution of compounds adsorbed on a 1.5 × 15 cm Darco charcoal column.

A volume of 16 ml of water was added above the charcoal column in tube "A", and a volume of 405 ml of water was added to the mixing flask "B". The volumes and concentrations of solvents added to the top flask "C" were as follows (the solution in the mixing flask "B" was not changed between new solvents):

First 300 ml: conc. aqueous $NH_3$–ethanol–$H_2O$ (5:1:13, v/v/v)

Second 300 ml: conc. aqueous $NH_3$–ethanol–$H_2O$ (5:5:13, v/v/v)

Third 300 ml: conc. aqueous $NH_3$–*n*-propanol–$H_2O$ (16:11:26, v/v/v)

Fourth 300 ml: conc. aqueous $NH_3$–*n*-propanol–*n*-butanol–$H_2O$ (14:12:7:21, v/v/v/v)

A flow rate of 0.25 ml/min was used throughout the elution.

## RESULTS

The elution peaks for some pyrimidines and purines and their nucleosides and nucleotides are shown in Fig. 2. The percentage recovery after adsorbing a known quantity of the compound on the charcoal was calculated from the area under the curve and the $\varepsilon_{260}$ value for the compound in alkaline solution. The precentage of recovery for each compound is shown in Table II.

TABLE II

RECOVERY OF COMPOUNDS FROM A 1.5 × 15 cm CHARCOAL COLUMN

| *Compound* | *Micromoles adsorbed* | *% Recovery* |
|---|---|---|
| Uridine-5′-phosphate | 2 | 88 |
| Uracil | 3 | 98 |
| Uridine | 2 | 97 |
| Cytidine | 3 | 77 |
| Adenosine | 2 | 72 |
| Adenine | 2 | 73 |
| Thymidylic acid | 3 | 65 |
| Adenosine-5′-phosphate | 3 | 95 |
| Thymidine | 3 | 99 |

## ACKNOWLEDGEMENT

This work was done under a predoctoral fellowship from the Public Health Service, U. S. Department of Health, Education, and Welfare.

## SUMMARY

A method has been described for the chromatography of purines, pyrimidines, and their nucleosides and nucleotides on activated charcoal. Spectrophotometric grades of *n*-butanol and *n*-propanol, used in the elution procedure, were prepared by treatment of the alcohols with sodium borohydride. In our laboratory charcoal chromatography proved more effective than other methods for purifying the acid-soluble uracil from rat liver slices in $^{14}C$-radioisotope experiments.

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# AN APPARATUS FOR CENTRIFUGAL ACCELERATION OF PAPER CHROMATOGRAPHY

J. R. TATA AND A.W. HEMMINGS
*National Institute for Medical Research, Mill Hill, London (Great Britain)*

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The period of several hours generally necessary for the development of paper chromatograms can become a major handicap in biochemical problems involving the use of radioactive isotopes of short half-lives. Recently, acceleration of paper chromatography by the application of centrifugal force has been described by McDONALD and his colleagues[1]. Their method consists of revolving horizontally a circular sheet of filter paper while the developing solvent is delivered continuously in a fine jet near the centre of the disc. Circular chromatograms tend to produce arc-shaped patterns, the size of the arc being proportional to the $R_F$ of the substance. Our determinations of radioactivity were made in an automatic strip counter which carries strips 2.5 cm in width. The spreading mentioned above would preclude any quantitative assessment of the radioactivity on such a cut from the centre of a circular chromatogram. A constant rate of flow of solvent is essential for satisfactory chromatographic separation and the difficulty in regulating solvent feed from an external reservoir might be an additional source of error. Both the above objections have been eliminated in our apparatus, described below. The circular filter paper sheet has been replaced by paper strips laid out in the form of independent "wheel spokes" while a wad of filter paper soaked in the solvent and placed in the development dish is all that is necessary for a solvent reservoir.

## APPARATUS

The apparatus consists of a flat, circular aluminium dish, 50.0 cm in diameter and 5.0 cm deep, mounted on bearings and rotated by means of a variable speed electric motor through a belt drive. The dish is coated on the inside with "Araldite" coating resin 985/C3 to prevent corrosion. At the centre of the dish is fixed an aluminium cup to hold a stack of 150–200 filter paper (Whatman No. 1 or 3) discs, 7 cm in diameter, which act as the reservoir. An adaptor to hold discs of larger diameter could be fitted to this cup. An annular ring of thick filter paper (Whatman No. 3, or 3 MM), soaked in the developing solvent, is placed at the bottom of the dish and around the inner cup to maintain a saturated atmosphere. The filter paper strips (60 × 2.5 cm) bearing the samples to be analysed are held in position by clamping a 0.5 cm thick Perspex lid to the rim of the dish which is 2.6 cm wide. A good seal is obtained by a ring of "Rubazote" 1.0 cm in thickness glued under the Perspex lid. The contact between the centre of the paper strips and the filter paper discs of the solvent reservoir is made by pressure from a small Perspex disc (5.5 cm in diameter and 0.25 cm thick) joined under the centre of the lid. A small length (3.5 cm) of capillary tubing surrounded by a Perspex tube is inserted through a hole in the centre of the lid and pressure disc to be used for introducing additional amounts of solvent after the dish has been sealed. A diagrammatic view of the apparatus is given in Fig. 1.

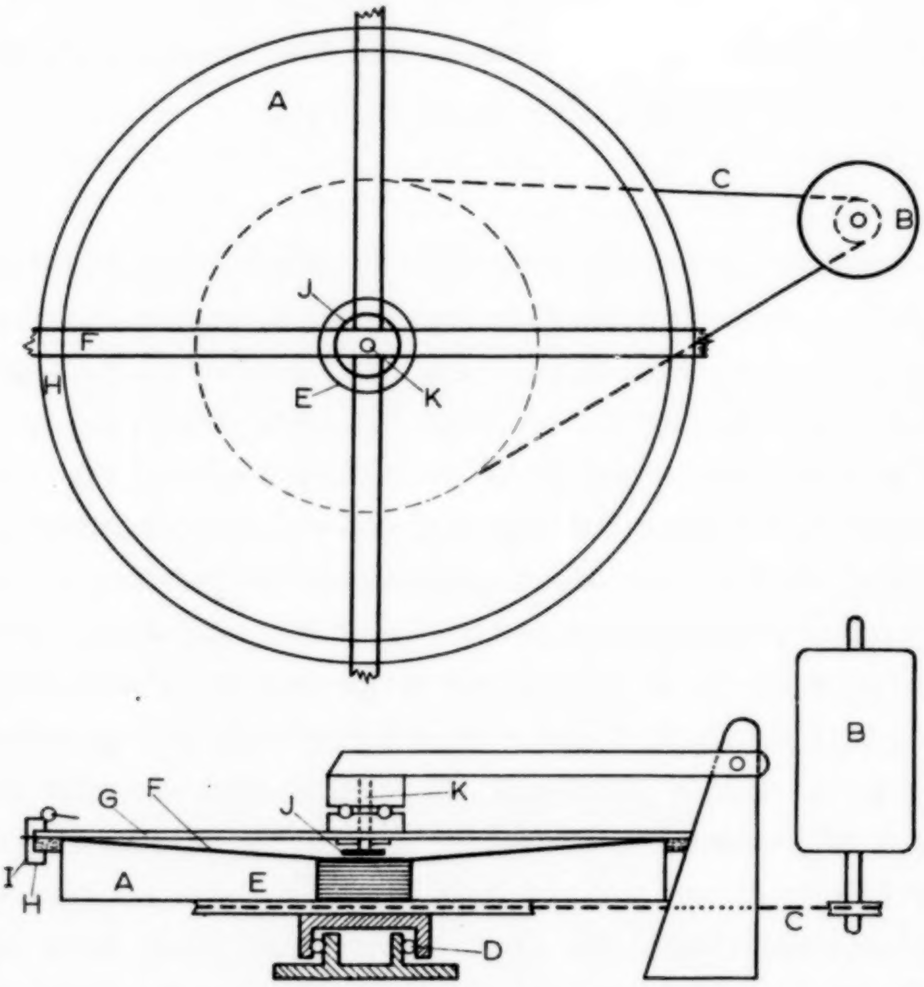

Fig. 1. Top and side views of the apparatus. A: aluminium dish; B: motor; C: belt; D: bearings; E: inner cup for filter paper solvent reservoir; F: chromatogram strips; G: Perspex lid; H: dish rim; I: clamp; J: Perspex disc; K: hole for capillary tubing.

## METHOD

The strips are prepared by applying and drying the mixture to be analysed at 7 cm on either side of the centre of the dish. To the wad of filter paper discs in the reservoir is added enough solvent (about 35–50 ml) to saturate it but not enough for the accumulation of any free liquid. The filter paper strips are laid diametrically across the dish and overlapping one another over the top of the reservoir. Up to 12 strips (and hence 24 samples) can be run simultaneously, without any contact between the points of application of samples or any part of the strip beyond 3–4 cm from the centre. A wad of 15 Whatman No. 1 filter paper discs, 7 cm in diameter, are soaked in the solvent and laid on top of the strips directly above the reservoir. The strips are maintained in a taut position by pulling them at both ends while the lid is being clamped on. An additional 5–7 ml of solvent are slowly introduced on to the top layer of filter paper discs through the capillary tubing in the central Perspex tube; the latter is then sealed with a rubber bung. Saturation of the atmosphere in the dish with solvent vapours is reached in 5–10 min by which time the solvent front on the strips has nearly reached the point of application of samples. The dish is then rotated at speeds varying between 150–400 r.p.m. for 15 to 60 min after which the strips are removed, dried and cut into two at the centre. If the samples are radioactive, the radioactivity distributed along the strip is directly measured with an automatically scanning ratemeter device and without any trimming of the filter paper. Later, the position of markers or carriers is determined by appropriate staining reactions described elsewhere[2].

## RESULTS

### (*a*) *Adsorption chromatography*

Most of the examples given by McDonald *et al.*[1] concerned adsorption rather than partition chromatography; the results of similar trials carried out with our apparatus, in order to compare the two methods, are shown in Table I. For this purpose, a mixture of bromophenol blue (BPB), methyl red (MeR) and methyl orange (MeO) was resolved into 3 components, using 0.075 $M$ veronal buffer, pH 8.6. It will be seen that centrifugal acceleration had little effect on the $R_F$ of the 3 dyes. Also, the constancy in $R_F$ values in our trials is comparable to that described by McDonald *et al.*[1] for chromatography on circular filter paper.

TABLE I

THE EFFECT OF INCREASING CENTRIFUGAL FORCE ON THE SEPARATION OF BROMOPHENOL BLUE, METHYL RED AND METHYL ORANGE WITH 0.075 $M$ VERONAL BUFFER, pH 8.6

| *Speed r.p.m.* | *Time run min* | *Length of chromatogram cm* | *$R_F$ values of* | | |
|---|---|---|---|---|---|
| | | | *MeO* | *MeR* | *BPB* |
| 0 | 300 | 8.9 | 0.26 | 0.63 | 0.89 |
| 200 | 15 | 4.8 | 0.25 | 0.62 | 0.89 |
| | 30 | 5.6 | 0.25 | 0.66 | 0.92 |
| | 45 | 6.9 | 0.28 | 0.67 | 0.93 |
| 400 | 15 | 5.1 | 0.26 | 0.66 | 0.90 |
| | 30 | 6.5 | 0.29 | 0.68 | 0.93 |
| | 45 | 7.7 | 0.29 | 0.67 | 0.91 |

### (*b*) *Partition chromatography*

The acceleration of partition chromatography was the main purpose in designing this apparatus, especially as applied to the separation of halogenated tyrosines, thyronines and their derivatives. In general, better results were obtained with acidic or neutral solvent systems than those equilibrated with ammonia. Some typical results obtained with the accelerated separation of radioactive bromide, 3,5-dibromotyrosine, 3,5,3′,5′-tetrabromothyronine, iodide, 3,5-diiodotyrosine and thyroxine in *n*-butanol–acetic acid–$H_2O$ as the solvent system* are presented in Fig. 2. Comparison of the patterns obtained with conventional ascending chromatography and the centrifugally accelerated process confirm that a shorter length of chromatogram and an increased rate of flow of solvent did not appreciably alter the $R_F$ values of these halogenated substances.

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* Bromide-82 and iodide-131 were obtained from A.E.R.E., Harwell; $^{82}$Br-labelled tetrabromothyronine was synthesized by a modification of Yagi's method[3] and $^{131}$I-labelled diiodotyrosine and thyroxine were obtained from Abbot Laboratories, Inc., Oak Ridge, Tenn.

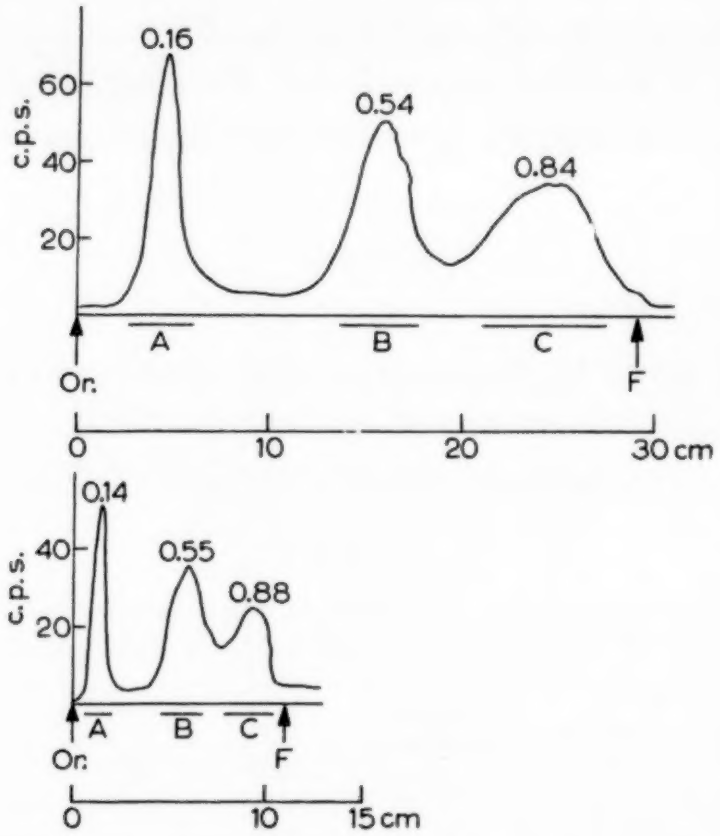

Fig. 2. Distribution of radioactivity of $^{82}$Br-labelled substances separated by conventional ascending (top) and centrifugally accelerated (bottom) paper chromatography. Solvent: *n*-butanol–acetic acid–water (78:10:12). A = bromide ion; B = 3,5-dibromo-L-tyrosine; C = 3,5,3′,5′-tetrabromo-L-thyronine. The figures above the peaks represent their respective $R_F$ values. Or = point of application of the mixture; F = solvent front. Chromatogram development times: 16.5 h for conventional chromatography and 22 min for centrifugal accelerated procedure. Time taken for recording of radioactivity = 62 and 23 min respectively.

In most cases, accelerated separation actually resulted in sharper resolution; at the same time, the shorter centrifugal chromatograms resulted in an important reduction in the time necessary for measuring radioactivity.

## DISCUSSION

With the apparatus described above it is possible to reduce the development time of chromatograms from several hours to a few minutes. Centrifugal acceleration of both adsorption and partition chromatography with this apparatus does not result in any distortion of patterns normally obtained with conventional chromatography. The design of our apparatus makes it more convenient to operate and more suitable for separation of radioactive materials than the apparatus for chromatography on circular sheets of filter paper. Besides the ease of handling paper strips (now available commercially in various sizes) arranged in the form of spokes of a wheel, the risk of contamination of radioactive samples due to sideways diffusion is completely eliminated. The solvent reservoir remains in the development dish and the system of filter paper discs soaked in the solvent ensures a constant and reproducible rate of flow of the solvent. Another advantage of our design of the solvent reservoir is that the starting gravitational force applied to draw the solvent can be varied by varying the diameter of the filter paper discs in the reservoir.

Results presented above show that centrifugal acceleration could be profitably applied in the chromatographic separation of substances labelled with radioisotopes of short half-lives. In this way, more information has been obtained on enzymic debromination of bromothyronines than has been hitherto possible[4]. As an actual example, chromatographic analysis of substances labelled with $^{82}$Br ($T_{1/2}$ = 35.9 h) by the conventional ascending method required 16 h for development and 24 h for measuring the distribution of radioactivity in 24 strips (automatic scanning at 7.5 cm/h) making a total of 40 h. With centrifugal chromatography the same operation was completed in 8.5 h (30 min development time and only 8 h for radioactivity measurements because of smaller chromatograms). This reduction in the time required for chromatographic analysis has meant a higher "workable" life for $^{82}$Br; it has also enabled us to increase the number of sequential experiments performed with $^{131}$I-labelled thyroid hormones and analogues. The advantage of speed is not restricted to chromatography of radioactive substances but could also be valuable in the separation of other materials, notably antibiotics and unstable substances.

## SUMMARY

The design and manipulation of an apparatus for centrifugal acceleration of paper chromatography has been described. The main novel features consists of using filter paper strips instead of circular sheets and a constant flow solvent reservoir enclosed in the chromatography tank.

Our arrangement is particularly suited for the quantitative analysis of materials labelled with radioactive isotopes of short half-lives and examples are given of the separation of some $^{82}$Br- and $^{131}$I-labelled substances. Advantages of the above method over circular paper chromatography are discussed.

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# MOLECULAR SIEVES AS SUBTRACTORS IN GAS CHROMATOGRAPHIC ANALYSIS

## II. SELECTIVE ADSORPTIVITY WITH RESPECT TO DIFFERENT HOMOLOGOUS SERIES

N. BRENNER, E. CIEPLINSKI, L. S. ETTRE AND V. J. COATES
*The Perkin-Elmer Corporation, Norwalk, Conn.* (*U.S.A.*)

(Received May 19th, 1959)

The use of synthetic zeolites of the "molecular sieve" (Linde Air Products Company, Division of Union Carbide and Carbon Corp.) type[1] as column packing material for gas chromatography is well recognized[2–6]. The unique ability of the 5 Å and 13 X types for the separation of oxygen and nitrogen, as well as other light gases, has brought them into widespread use. Molecular sieves, however, also have another useful property for gas chromatography.

In a previous publication[7], selective retention of molecular sieves type 5 Å of *n*-paraffins in mixtures of *n*-paraffins, isoparaffins, cycloparaffins and aromatics was described. This selective retention of *n*-paraffins has been used to aid in qualitative identification of components in complex hydrocarbon mixtures[8].

It was noted in one publication[7] that molecular sieves also show similar selective irreversible adsorptivity with respect to other homologous series. The object of the present work was to determine, which compounds and homologous series are irreversibly adsorbed on the sieve column and which are passed and furthermore, the temperature limitations of the system.

### EXPERIMENTAL

The experiments were carried out with a Perkin-Elmer Model 154-C Vapor Fractometer. A 50 cm long, 1/4 in. diameter column was packed with 20–60 mesh "Molecular Sieve 5 Å" and "Molecular Sieve 13 X" respectively and installed into the chromatograph. The temperature of operation was 100°. Helium was used as carrier gas and a 2 lb/in.$^2$ pressure was sufficient to maintain a 60 cm$^3$/min flow.

Successive samples of about 2 $\mu$l of each compound to be tested were run through the system and the eluted peaks recorded on a 5 mV potentiometer recorder. The components were of high purity ACS, Phillips Research Grade or equivalent.

The samples were selected wherever possible to include the lightest member of a homologous series, the next heavier member and at least one relatively heavy representative member.

## RESULTS AND DISCUSSION

The compilation of the investigations on the column filled with molecular sieve 5 Å is given in Tables I and II: Table I gives the materials which pass through molecular sieve 5 Å without adsorptive loss and Table II shows the materials which were found to be completely adsorbed under the described conditions.

TABLE I

COMPONENTS PASSED THROUGH MOLECULAR SIEVE 5 Å COLUMN

| *Group* | *Components tested* |
|---|---|
| Isoparaffins | Isobutane, Isopentane, 2,3-Dimethylbutane |
| Aromatic hydrocarbons | Benzene, Toluene, *m*-Xylene |
| Cycloparaffins | Cyclopentane, Cyclohexane |
| Iso-olefins | Isobutylene, 2-Methylbutadiene-1,3 |
| Esters | Amyl formiate, Ethyl acetate, Ethyl propionate |
| Ketones | Acetone, Methyl ethyl ketone, Mesityl oxide |
| Halogenated hydrocarbons | Methylene chloride, Chloroform |
| Iso-alcohols | Isopropanol, Methylbutanol |
| Ethers | Diethyl ether, Di-isopropyl ether |
| Other compounds | Carbon monoxide, Oxygen, Nitrogen, Rare gases, Methane, Nitromethane, Carbon disulfide, Dimethyl sulfide, Thiophene |

TABLE II

COMPONENTS ADSORBED COMPLETELY ON MOLECULAR SIEVE 5 Å COLUMN

| *Group* | *Components tested* |
|---|---|
| Normal paraffins (except methane) | Propane, *n*-Butane, *n*-Hexane |
| Normal olefins | Ethylene, Propylene, Hexene-2 |
| Normal alcohols | Methanol, Ethanol, *n*-Butanol |
| Aldehydes | Acetaldehyde, Propionaldehyde, Isovaleraldehyde |
| Acids | Formic acid, Propionic acid |

The other types of molecular sieves do not behave similarly, since their pore openings are different. The molecular sieve 4 Å (pore opening of 4 Å diameter) adsorbs only ethane of the *n*-paraffins, with elution of propane and higher members. Of the *n*-olefins ethylene and propylene are adsorbed on the molecular sieve type 4 Å but the butenes and higher are not. The situation is similar with oxygenated compounds: *e.g.* the *n*-alcohols from *n*-butanol pass through this material, because their molecules are larger than 4 Å. Whereas, because of the very large pore openings (about 13 Å), the molecular sieve type 13 X adsorbs practically all organic compounds which are given in Table I (these components pass through the 5 Å molecular sieve column). Oxygen, nitrogen, carbon monoxide and methane are, furthermore, not adsorbed by this column.

The above illustrates that because of the variety of molecular sieves it is possible to select a given type which retains individual compounds in a sample, while the others pass through the column.

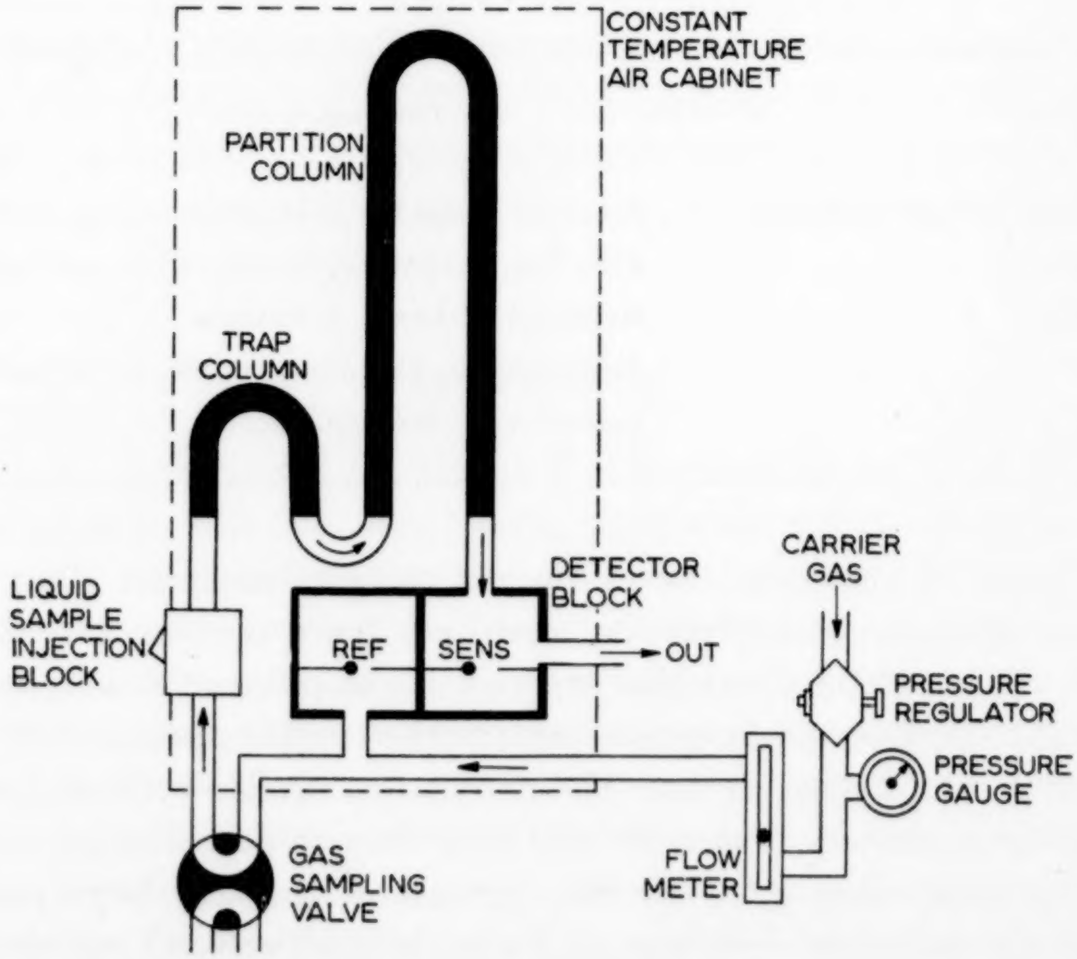
### *Application of the specific adsorption characteristics of molecular sieves*

The specific adsorption characteristics of molecular sieves offer in practice two advantages:

1. In analysis of complex mixtures, containing various series of homologs, the removal of one of these series will serve to quantitatively identify the series, each of the members of the series (since elution time is proportional to carbon number) and finally, indicate the possible identity of non-adsorbed components.
2. The removal of an adsorbable component from a system may permit observation and measurement of peaks otherwise obscured by overlap.

In these applications, the molecular sieve column is to be placed in series with a standard partition column, as shown in Fig. 1. In this case, mixtures of components were injected into the unit and passed first through the molecular sieve column. Here some components were adsorbed and those which passed through were separated by the partition column in the standard manner.

The analysis of complicated hydrocarbon mixtures is a good example for using this technique. In an earlier publication[7] the fractogram of *n*-paraffin subtraction by


CONSTANT
TEMPERATURE
AIR CABINET
PARTITION
COLUMN
TRAP
COLUMN
LIQUID
SAMPLE
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OUT
CARRIER
GAS
PRESSURE
REGULATOR
PRESSURE
GAUGE
GAS
SAMPLING
VALVE
FLOW
METER

Fig. 1. The use of a molecular sieve column as trap column in the gas chromatograph.

molecular sieves type 5 Å was given. WHITHAM[8] used the same method for the determination of the *n*-paraffins in heavier petroleum fractions.

In other cases, the removal of a component is necessary, which partly overlaps other components in the fractogram. The right part of Fig. 2 shows the fractogram of a mixture containing isopropyl ether, propionaldehyde, acetone, ethyl acetate and ethanol, analyzed on a standard partition column[*]. As shown, the acetone peak is partly overlapped by the propionaldehyde peak. Using a short molecular sieve 5 Å column in series, the propionaldehyde peak (and the peak of ethanol) is completely removed.

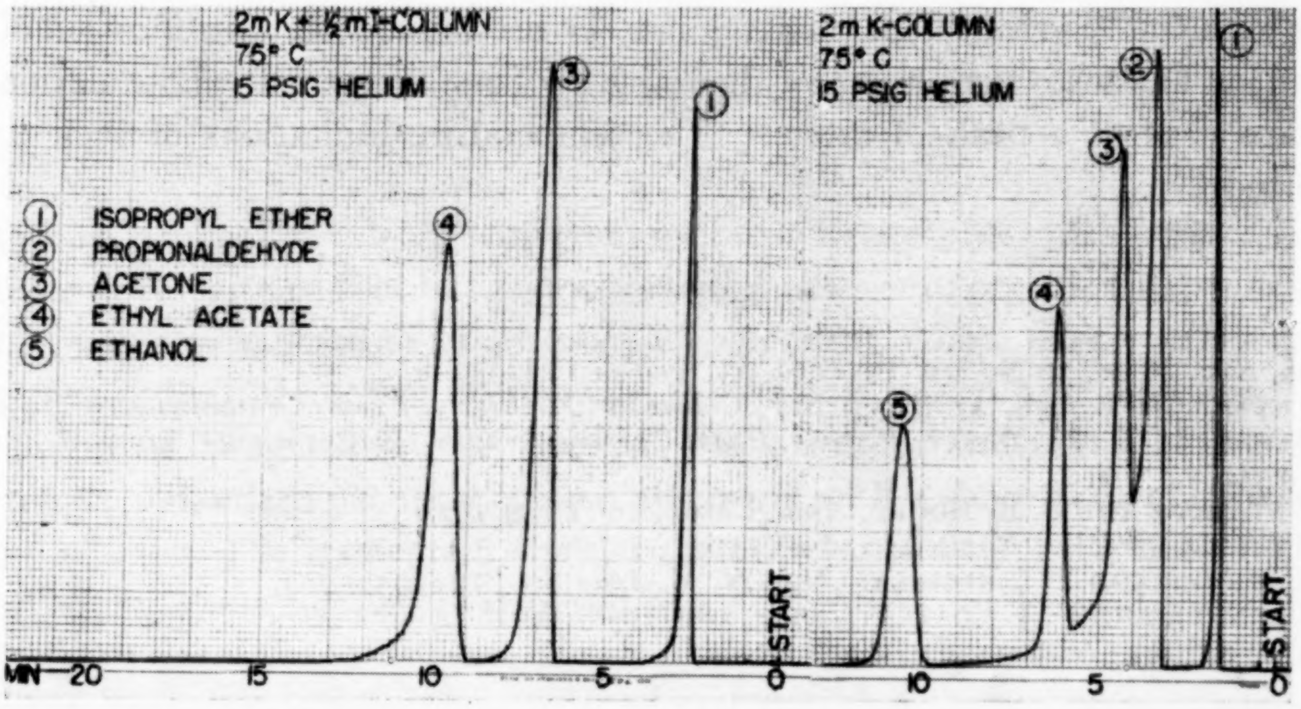

Fig. 2. Analysis of a mixture of: 1, isopropyl ether (16.7%); 2, propionaldehyde (33.2%); 3, acetone (16.7%); 4, ethyl acetate (16.7%); and 5, ethanol (16.7%), at 75° and 15 p.s.i.g. helium inlet pressure. Column: at the right analysis, 2 m K; at the left analysis, ½ m I + 2 m K.

### *Temperature limitations*

As shown, the temperature used in this investigation was 100°. Operating at temperatures below 100°, the adsorptive effects of the sieves will result in increasing the retention time of components which would pass through rapidly at higher temperatures. Analyzing *e.g.* a hydrocarbon mixture containing olefins, the iso-olefins such as isobutene are strongly held at 75° or lower even though this class of compound should be eluted. The molecular sieves should therefore be used at high temperatures for subtractive purposes in order to minimize retention time of components which are not irreversibly held. In the case of analyses of light components the sieve column may be heated separately by resistance wire to a level higher than that of the necessarily cool analyzing column.

In the subtraction of *n*-paraffins, temperatures as high as 130° have been used. Even at this elevated temperature, ethane and propane are held for long periods, while components boiling as high as *o*-xylene are eluted without delay. It might be mentioned that carbon dioxide is eluted at this temperature in 10 minutes.

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[*] The column designations of the Perkin-Elmer Corporation are the following: Column I: Molecular sieve 5 Å column (20–60 mesh); Column K: 30 weight-% polyethylene glycol (Carbowax 1500) on Chromosorb 30–60 mesh.

## SUMMARY

The use of Linde Company "Molecular Sieves" in gas chromatography to irreversibly adsorb particular components was discussed. The behavior of various homologous series and special components of general interest on columns run at 100° is shown. Since those compounds not adsorbed pass through with virtually no retention time, they may be conventionally separated by partition on adsorption columns placed in series with the "Sieves". This property may be used to aid the identification of components in unknown samples or for the removal of one component whose elution band overlaps that of a peak of interest. The temperature limitations of their use are discussed.

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# MOLECULAR SIEVES AS SUBTRACTORS IN GAS CHROMATOGRAPHIC ANALYSIS

## III. THE SECONDARY EFFECT OF THE MOLECULAR SIEVE TRAP COLUMN

L. S. ETTRE AND N. BRENNER
*The Perkin-Elmer Corporation, Norwalk, Conn. (U.S.A.)*

(Received May 19th, 1959)

A previous publication[1] dealt with the selective irreversible adsorptivity of the molecular sieves with respect to various homologous series of organic compounds. For illustration of the removal of an adsorbable component, whose peak overlaps partly or completely that of another component, the fractograms of a mixture containing isopropyl ether, propionaldehyde, acetone, ethyl acetate and ethanol were given, analyzed on a standard partition column alone and with a short molecular sieve 5 Å column in series. The fractogram showed that the propionaldehyde peak (and the peak of ethanol) was completely removed.

One purpose of the investigations was to show the analysis of a small amount of acetone in propionaldehyde, since in a normal partition system these components overlapped almost completely. Upon injection into a series column system of molecular sieve and partition column, the unexpected result was found to be that neither component was eluted. This result was confirmed by many repetitions.

This observation induced us to investigate this phenomenon in detail.

### EXPERIMENTAL

The experiments were carried out with a Perkin-Elmer Model 154-C Vapor Fractometer. A 50 cm long, ¼ in. diameter column was packed with 20–60 mesh "Molecular Sieve 5 Å" and placed in series with a 2 m long standard partition column containing 30 weight-% polyethylene glycol (Carbowax 1500) on Chromosorb 30–60 mesh*. Fig. 1 in our recent publication[1] showed the schema of the instrument.

### RESULTS AND DISCUSSION

We prepared propionaldehyde–acetone mixtures with different acetone concentrations and analyzed them under the given conditions with the gas chromatograph. At higher acetone concentrations we regularly obtained, as expected, the acetone peak, and the propionaldehyde was adsorbed completely on the trap column. It was found, however, that low concentrations of acetone in propionaldehyde would give no peak at all: only levels above 5 % produced any peak response. However, by changing the matrix material from propionaldehyde to ethyl acetate, full response to 4.8 % acetone concentration was restored.

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* The column designations of the Perkin-Elmer Corporation are the following: molecular sieve 5Å column: column I; polyethylene glycol column: column K.

Fig. 1 illustrates the phenomena mentioned above. Runs A and B show the results of two parallel analyses, running a mixture of 4.8 % acetone in propionaldehyde.

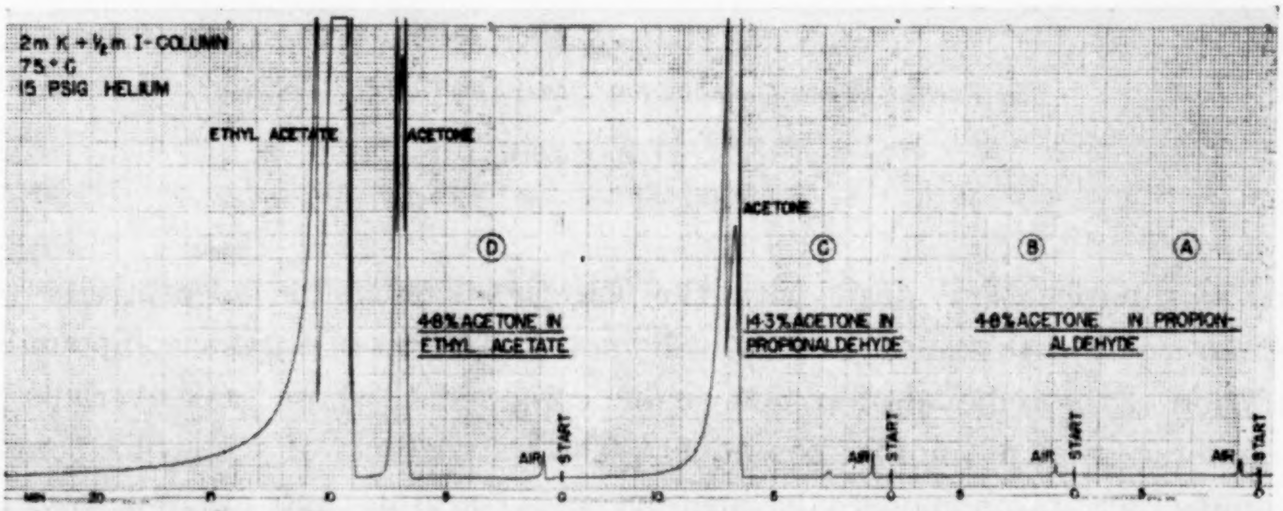


Fig. 1. 2 m K + ½ m I column, 75°, 15 p.s.i.g. helium inlet pressure. The samples: A and B, 4.8 % acetone in propionaldehyde; C, 14.3 % acetone in propionaldehyde; D, 4.8 % acetone in ethyl acetate.

As shown, the analysis would give no peak at all. On the other hand, 4.8 % acetone in ethyl acetate gives an excellent response (run D). Run C shows the analysis of a mixture of 14.3 % acetone in propionaldehyde.

These results suggested that somehow in the presence of propionaldehyde the acetone reacted to form an adsorbable product. In this case, however, on analyzing mixtures with higher acetone concentrations, a certain amount of acetone would subtract from the apparent amount present in each mixture.

For investigation of the value of this supposition, two mixtures were prepared, one of 10 % acetone in propionaldehyde, the other 10 % acetone in ethyl acetate, and analyzed successively through the system. Fig. 2 shows the fractogram of the two analyses. As shown, the response to 10 % acetone is almost exactly twice as great—as seen from the sensitivity notations—for acetone in ethyl acetate as it is for acetone in propionaldehyde. This indicates that about 5 % of the acetone in the propionaldehyde does not elute, which confirms the earlier "threshold" finding.

This secondary adsorption effect was observed in other cases too, *e.g.* in the case of water solutions, which is illustrated with another examination.

The analysis of organic solvents dissolved in water is a general problem and in earlier work[2] gas chromatography was used for the analysis of very small amounts of isopropyl ether (below 0.5 %) in water. This method, however, has two difficulties: because of the large amount of water, the sample must be analyzed at 100° and even at this temperature, the water peak shows severe tailing. Therefore, the analysis takes

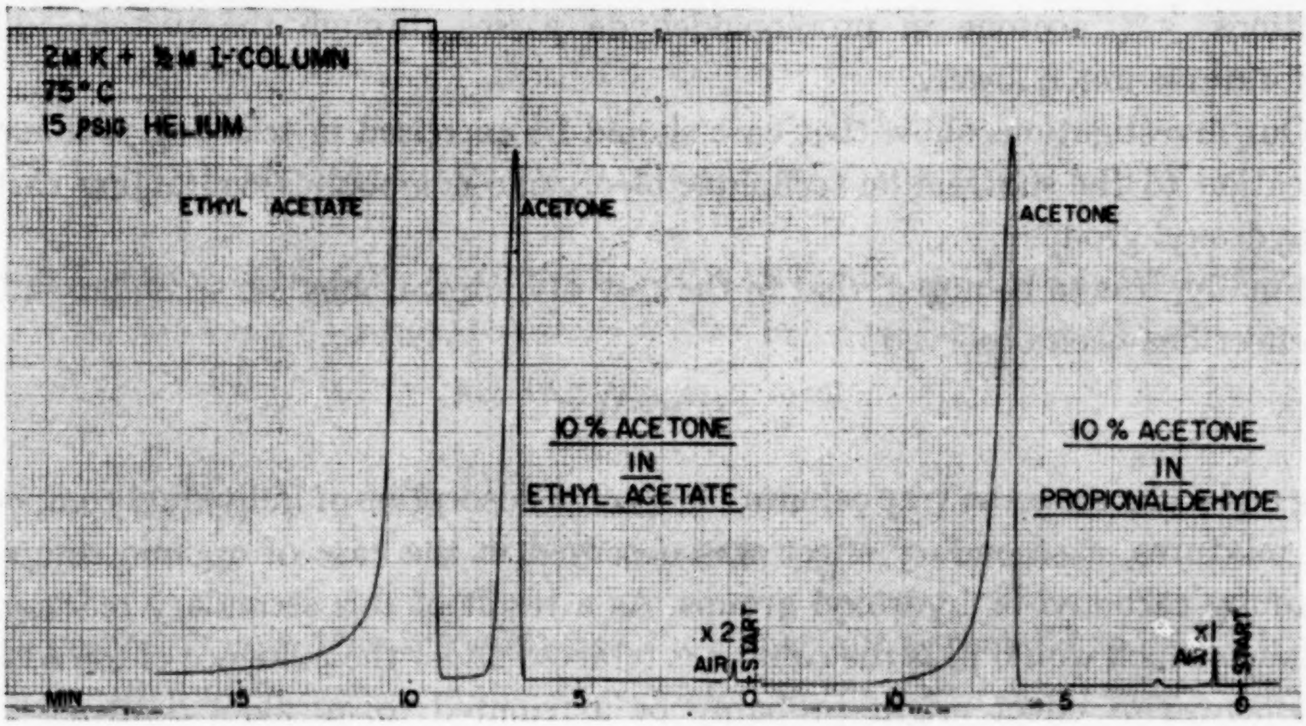
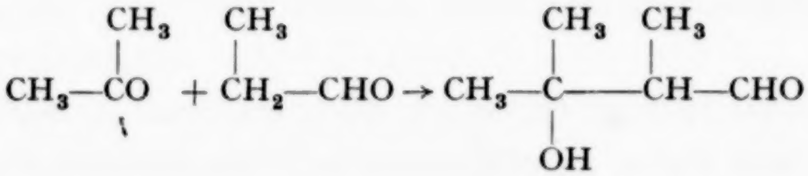


Fig. 2. 2 m K + ½ m I column, 75°, 15 p.s.i.g. helium inlet pressure. Sample at the right analysis: 10 % acetone in propionaldehyde; at the left analysis: 10 % acetone in ethyl acetate.

a long time. We tried to carry out the analysis using a trap column containing molecular sieve 5 Å, with the supposition that in this case the water would be adsorbed on this column. Actually the water peak disappeared but—similar to the analysis of acetone in propionaldehyde—no peak was obtained for the isopropyl ether either.

For the interpretation of the phenomena described above, we suppose the following:

(a) Because the molecular sieves are basic substances, the passage of acetone–propionaldehyde mixtures over this material would tend to create suitable conditions for an aldol condensation reaction. Since the resulting product would be an aldehyde



$$CH_3\text{—}\overset{\displaystyle CH_3}{\overset{|}{C}}O + \overset{\displaystyle CH_3}{\overset{|}{C}}H_2\text{—}CHO \rightarrow CH_3\text{—}\underset{\displaystyle OH}{\underset{|}{\overset{\displaystyle CH_3}{\overset{|}{C}}}}\text{——}\overset{\displaystyle CH_3}{\overset{|}{C}}H\text{—}CHO$$

it would be adsorbed on the sieve. The concentration of 5 % acetone and 95 % propionaldehyde would correspond to the equilibrium on the given conditions.

(b) In the case of the isopropyl ether–water mixture, secondary hydrogen bonding between the adsorbed water and the ether cause the retention of the ether on the column.

The equilibrium conditions of the aldol condensation on the column are dependent on several parameters, *viz.* the temperature, the concentration of the acetone in the propionaldehyde, etc. The effect of the temperature is double: on raising the temperature the conditions become more favorable, but at the same time, the retention time of acetone is shorter on the molecular sieve column and according to this, the contact time is shorter, which results in a lower yield of the condensation product. Thus, our examinations show that at 100° temperature but otherwise under the same conditions, 5 % acetone in propionaldehyde passes through the system and the equilibrium is much lower.

Our investigations show that care should be exercised in selecting problems for application to the subtraction technique of organic compounds containing carbonyl and hydroxyl groups.

Finally, it is to be noted, that in the case of hydrocarbons[3] no secondary adsorption effect has been observed.

## SUMMARY

Using molecular sieves as trap column for specific adsorption of individual components from mixtures, a secondary effect was observed in the case of organic compounds containing carbonyl or hydroxyl groups. As a result of this secondary reaction, the substance which would pass the column is retarted under the influence of the adsorbed component. This effect might probably be attributed to an aldol condensation or formation of secondary hydrogen bonds. These reactions could promote the basic character of the molecular sieves. In the case of hydrocarbons, no secondary adsorption effect has been noted.

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# ZONE MIGRATION IN PAPER CHROMATOGRAPHY

J. CALVIN GIDDINGS, GEORGE H. STEWART AND ARTHUR L. RUOFF*
*Department of Chemistry, University of Utah, Salt Lake City, Utah (U.S.A.)*

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## INTRODUCTION

The $R_F$ value has long been recognized as the important parameter in characterizing solute migration in chromatography. It is related to the partition of solute molecules between the mobile phase and the stationary phase. The equation which has been extensively used to describe this dependence is

$$R_F = \frac{A_M}{\alpha A_S + A_M} \tag{1}$$

where $A_M$ and $A_S$ are the respective cross sectional areas of mobile phase and stationary phase solvents. It will be noticed that the ratio $A_M/(\alpha A_S + A_M)$ in this equation is the ratio of the number of molecules in the mobile phase to the total number of molecules. In order that this may be considered equal to the true $R_F$ (the distance traveled by the center of the zone divided by the distance traveled by the solvent front) two assumptions must be made. They are:

1. The average solvent velocity at the spot location must be equal to the forward velocity of the front, and
2. The ratio $A_M/A_S$ at the spot location must remain constant irrespective of the distance migrated by the spot.

We have investigated these assumptions in the light of recent work on solvent flow in paper. Our conclusions are that assumption 1 is not true and that the local solvent velocity falls behind that of the front by a varying amount depending on the distance from the front. Concerning assumption 2, we find that this can be correct under the condition that the spot is started right at the solvent source and the flow is horizontal. Otherwise, we obtain a varying $R_F$, as has been commonly observed in ascending and descending flow, and when the spot is started a large distance along the paper[1-3]. The fact that $A_M/A_S$ is not constant along a paper strip has been often recognized in view of the concentration gradients found in the solvent concentration. This fact has not, however, been generally related to zone migration. An exception is found in the work of WOOD AND STRAIN[2] where these effects are theoretically considered. By virtue of recent work on solvent distribution in paper the theory of zone migration can now be given in quantitative form for any paper for which a single concentration profile is known. Experiments have been performed with Whatman 3 MM paper to evaluate the applicability of the theory.

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* Department of Mechanics and Materials, Cornell University, Ithaca, N.Y.

## THEORY

It has been established[4] that the flow of solvent in paper obeys the diffusion equation

$$\frac{\partial w}{\partial t} = D\frac{\partial^2 w}{\partial z^2} \tag{2}$$

where $w$, the relative concentration, is the local ratio of solvent to dry paper weight. The solvent weight is that obtained over and above any absorbed in the pretreatment of the paper. The effective diffusion coefficient, $D$, is a function of $w$. This makes equation (2) nonlinear. Despite the difficulties of obtaining solutions to this equation, a very useful property arises with the application of the Boltzmann transformation[5]. Thus, for a one-dimensional problem such as with a rectangular sheet or strip immersed on one end, the concentration $w$ is a function of the single variable $z/t^{1/2}$. Considering the fact that $z_f$, the distance to the solvent front, is merely the distance at which $w$ approaches zero, this fact explains the parabolic flow rate law

$$z_f^2 = \varkappa t \tag{3}$$

where $\varkappa$ is the flowrate coefficient. An equally significant deduction is that all concentration profiles in which $w$ is plotted against $z$ become identical when $w$ is plotted against the reduced distance $y = z/z_f$. This has been experimentally verified using water and $n$-butanol in Whatman 3 MM, 1 and 54 papers. The separate concentration profiles are illustrated in Fig. 1, and the reduced profile in Fig. 2. Each paper-solvent system will have a characteristic reduced profile. The concept of reduced profiles is an important simplification in calculating $R_F$ values, as will be shown. In addition to the

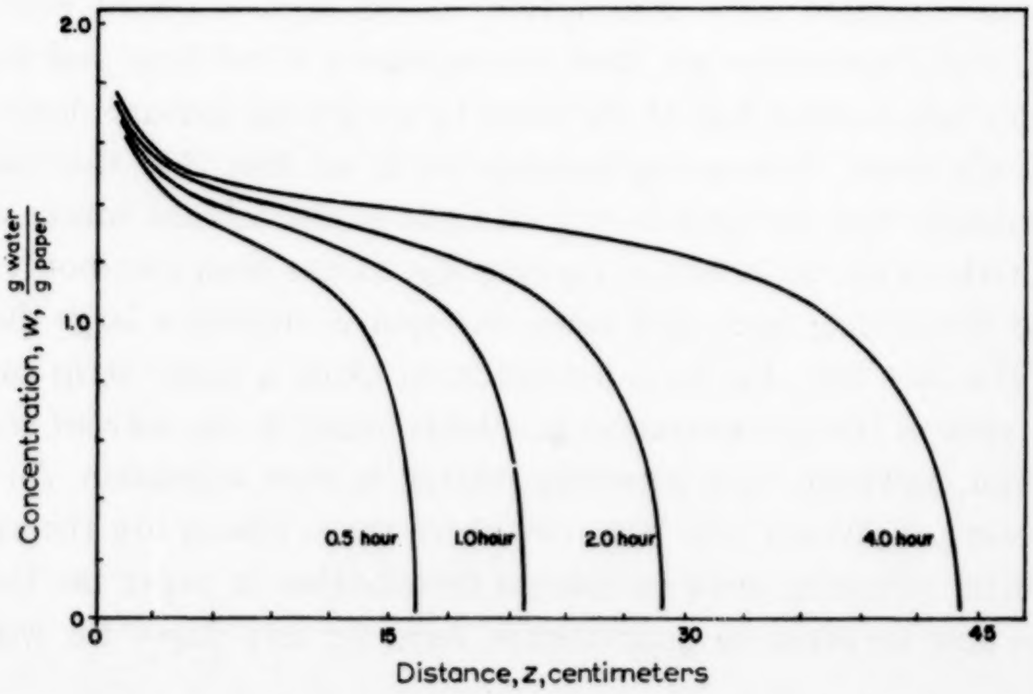

Fig. 1. Concentration profiles for Whatman 3 MM systems at various time intervals.

rectangular strips, there is good evidence that tapered strips, discs, discs with wicks, etc., all exhibit reduced concentration profiles after the passage of a short initiation period. This fact is of importance in circular chromatography, with and without wicks.

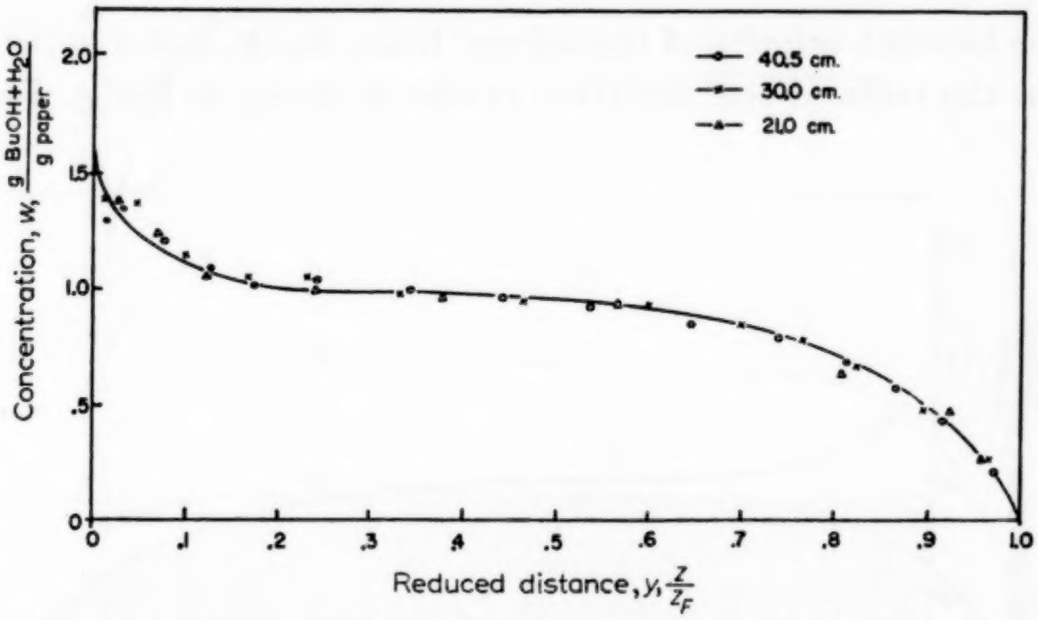

Fig. 2. Reduced concentration curve for linear, horizontal flow of *n*-butanol ($H_2O$ saturated) on Whatman 3 MM paper.

In order to describe zone migration it is necessary to know the fraction of molecules in the mobile phase as a function of the concentration $w$. This ratio, given in eqn. (1), will hereafter be referred to as $R$ since the ratio is not necessarily equal to the $R_F$ value. If we make the very reasonable assumptions that $A_M$ is proportional to $w$, and that $A_S$ is constant (this assumes an adequate pre-exposure to saturated vapor), it is possible to rewrite the ratio in (1) as

$$R = \frac{w}{w + c} \tag{4}$$

where

$$c = \frac{aw}{A_M/A_S} \tag{5}$$

the constant $c$ will vary from one paper–solvent system to another, but will remain essentially constant in a given system for either strips or discs.

Since a fraction $R$ of the molecules are in the mobile phase, the mean velocity, $\bar{u}$, of a zone will be

$$\bar{u} = Rv \tag{6}$$

where $v$ is the average value of the local solvent velocity. This velocity can be obtained as the ratio of the solvent flux, $q$, to the concentration $w$

$$v = q/w \tag{7}$$

the flux $q$ must be calculated in terms of the grams of solvent passing in one second through a normal, cross sectional area just large enough to contain 1 g of dry paper per cm of length (or equivalent units in another system). Substituting eqns. (7) and (4) into (6) we obtain

$$\bar{u} = \frac{q}{w + c} \tag{8}$$

As long as the flow process maintains a reduced concentration profile, the flux is given by[4]

$$q = v_f A = v_f \int_0^w y \, dw \tag{9}$$

where $v_f$ is the forward velocity of the solvent front, $dz_f/dt$, and $A$ is the shaded area obtained from the reduced concentration profile as shown in Fig. 3. The value of $A$ depends upon the reduced coordinate $y$. In the figure the $A$ is to be associated with a $y$ directly below the intersection (arrow) of the reduced profile and the area enclosure line.

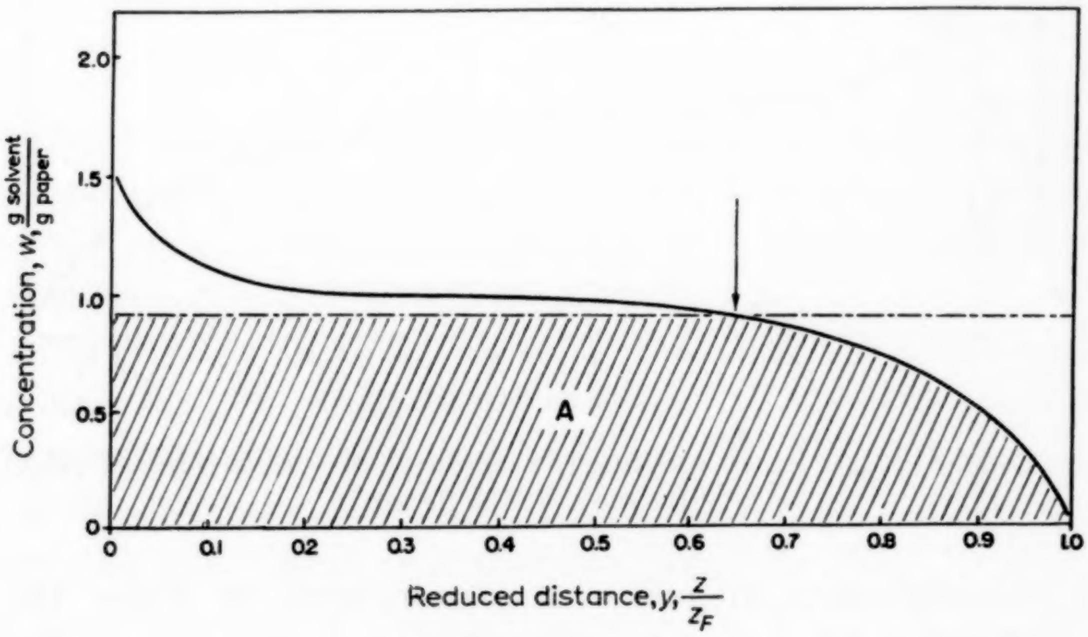

Fig. 3. The area term, $A = \int_0^w y \, dw$.

Combining eqns. (8) and (9) we have the ratio of the zone velocity to the solvent front velocity

$$\frac{\bar{u}}{v_f} = \frac{A}{w + c} \tag{10}$$

Since $A$ and $w$ depend only upon the reduced distance $y$, the above ratio depends only upon $y$ and the system constant $c$. This ratio of velocities is plotted in Fig. 4 using a water-saturated butanol solvent on Whatman 3 MM paper.

An interesting result can be obtained from eqn. (10) by considering the case $c = 0$. Here $R = 1$ and $\bar{u} = v$, the local solvent velocity.

$$\frac{v}{v_f} = \frac{A}{w} \tag{11}$$

This ratio is always less than unity for the rectangular sheets and strips now under consideration. In Fig. 3 the ratio $A/w$ is shown as the ratio of the shaded area to the area enclosed by the dotted line. This departs from unity more and more as $y$ becomes smaller as shown by the top line in Fig. 4. This fact by itself makes eqn. (1) incorrect as a description of $R_F$.

We wish now to examine the $R_F$ value on a horizontal paper strip and describe

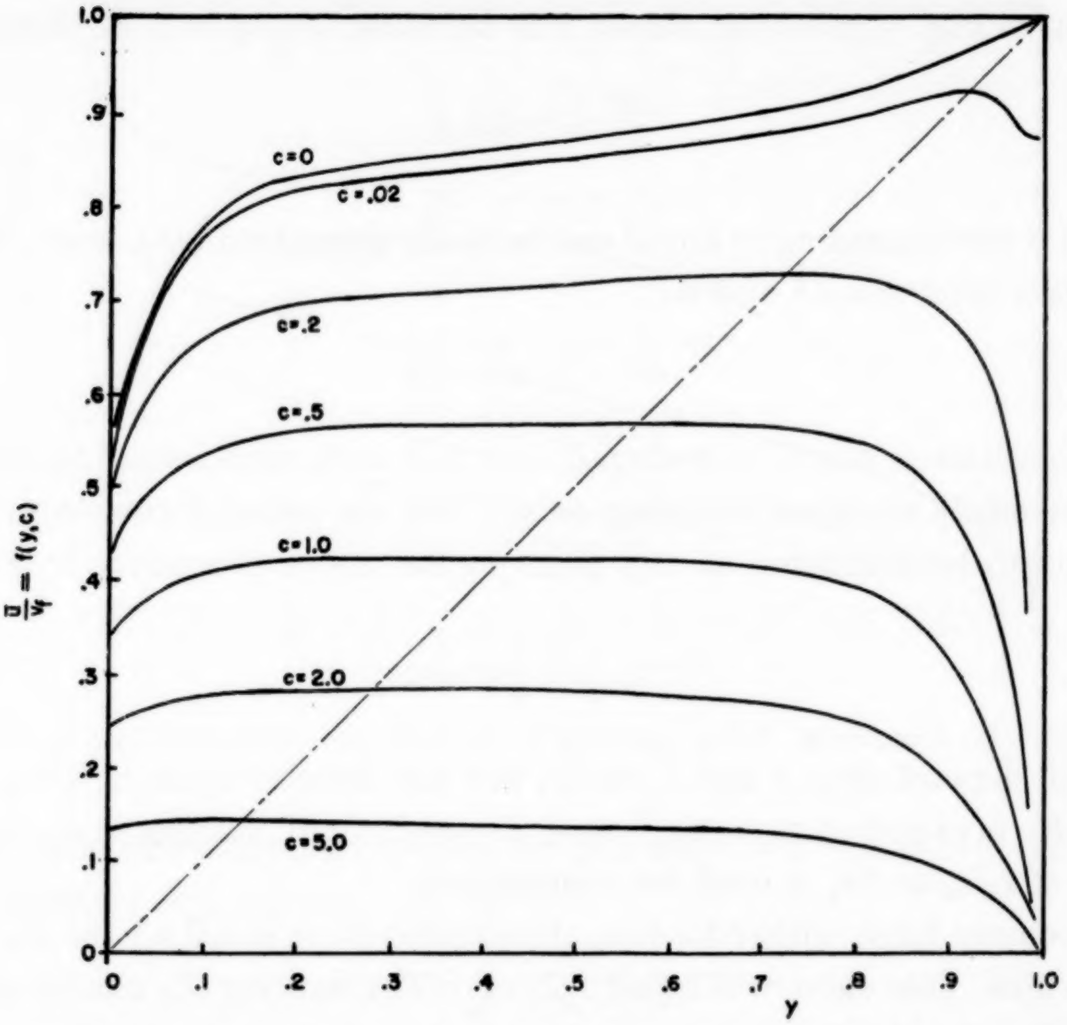

Fig. 4. Relative velocity function, as a function of fractional distance to the solvent front, $y$, for various values of the partition parameter, $c$, in a $n$-butanol ($H_2O$ saturated) Whatman 3 MM system.

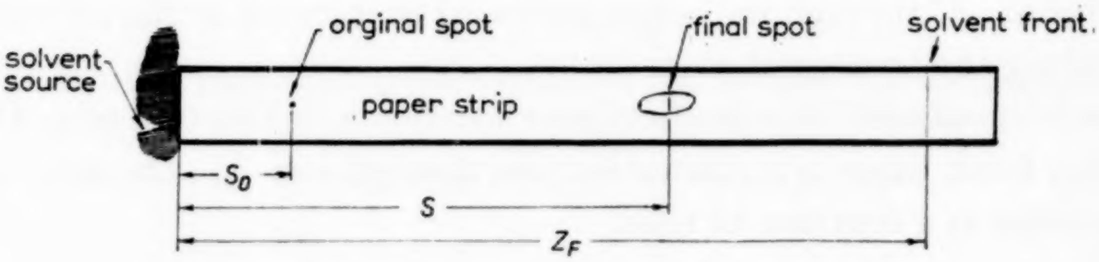

Fig. 5. The coordinates in paper chromatography.

this in the simplest way as a function of (i) the distance from the solvent source to the original spot location, and (ii) the extent of development. Fig. 5 illustrates the meaning of the various coordinates. In terms of these coordinates, $\bar{u} = \mathrm{d}s/\mathrm{d}t$ and $v_f = \mathrm{d}z_f/\mathrm{d}t$. The ratio of the two, $\mathrm{d}s/\mathrm{d}z_f$, is given in eqn. (10)

$$\frac{\mathrm{d}s}{\mathrm{d}z_f} = \frac{A}{w + c} \tag{12}$$

It is convenient to introduce the reduced coordinates (not to be confused with $y$) $S = s/s_0$ and $Z = z_f/s_0$. With this change we have

$$\frac{\mathrm{d}S}{\mathrm{d}Z} = \frac{A}{w + c} = \mathrm{f}\ (y,c) \tag{13}$$

where we have indicated that the ratio $A/(w + c)$ is a function only of $y$ and $c$.

Examination of Fig. 5, however, shows $y$ to be equal to $s/z_f = S/Z$. Hence

$$\frac{dS}{dZ} = f\,(S/Z,c) \tag{14}$$

The value of $S$ corresponding to any $Z$ can be easily generated by a series of steps with the small finite intervals $\delta S$ and $\delta Z$.

$$\delta S = f\,(S/Z,c)\;\delta Z \tag{15}$$

The initial condition is that $S = 1$ when $Z = 1$. It is to be emphasized that the function f $(y,c)$ can be easily obtained knowing only $c$ and the reduced concentration profile.

The $R_F$ can be evaluated at any point in the above procedure by the equation

$$R_F = \frac{s - s_0}{z_f - s_0} = \frac{S - 1}{Z - 1} \tag{16}$$

Its value will depend upon $c$ and $Z$ (or $S$), but not directly upon $s_0$. Thus for a given system the $R_F$ is expected to be the same, irrespective of changes in $s_0$, as long as the same value of $z_f/s_0$ or $s/s_0$ is used for comparison.

As $S$ becomes large (either for long developments or small $s_0$) the $R_F$ approaches a constant value. This value will equal $S/Z$, or $y$. The limiting $R_F$ can be obtained as a solution to the equation

$$R_F = f\,(R_F,c) \tag{17}$$

This value of $y = R_F$ can be obtained graphically from Fig. 4 using the 45° rule. Thus a straight line at 45° through the origin intersects each curve at the correct $R_F$ value, to be read along the horizontal axis.

The method outlined here predicts zone location as a function of $z_f$, the distance to the solvent front. Since $z_f$ is related to time through eqn. (3), the zone location can easily be written as a function of time.

## RADIAL FLOW

Strong experimental and theoretical evidence exists[4] which indicates that the concentration profiles obtained in radial (horizontal) flow can be reduced to a common profile after a short transient period. The reduced profile depends upon whether a wick is used or not, and upon the dimensions of the wick if one is used. Fig. 6 shows several reduced profiles for radial flow, one without a wick and the three others with wicks of varying length. It is significant that the profile becomes lower for longer wicks, providing the width remains constant. A long wick acts essentially as a large resistance to flow. For this reason discs with long wicks have the lower concentration profile shown in Fig. 6.

With the use of reduced concentration profiles in radial flow, zone migration can be calculated in much the same manner as before. Eqns. (4–8) are valid for flow in strips, radial flow, and flow in other geometries that may be used. The equation for

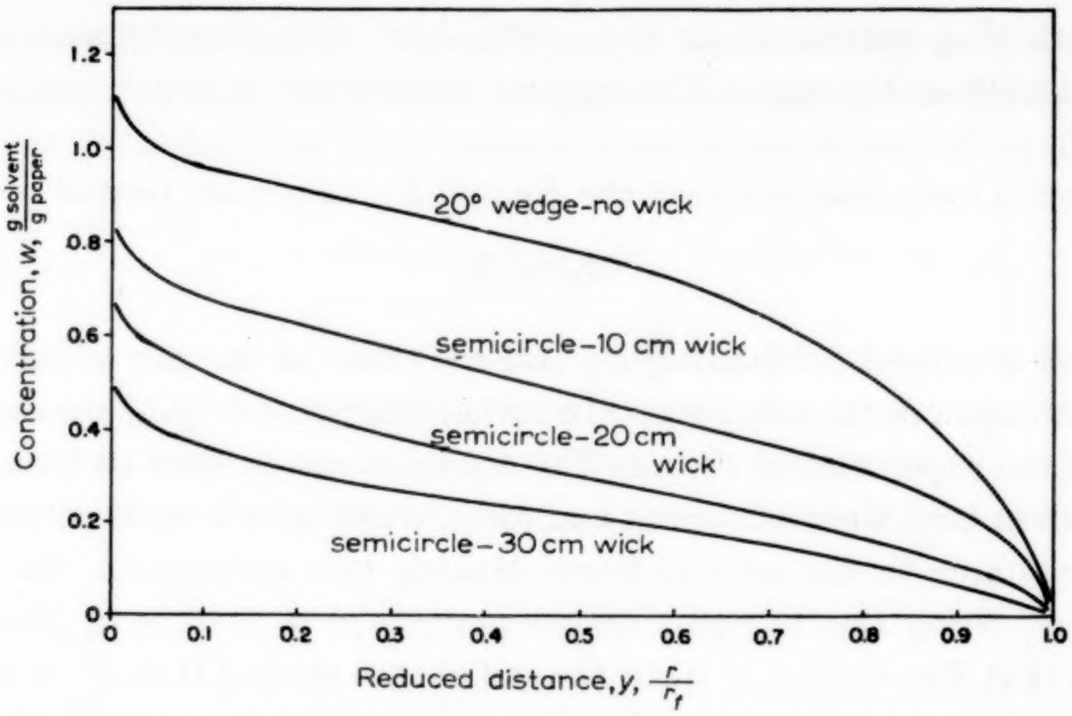

Fig. 6. Experimental concentration profiles; Whatman 3 MM, *n*-butanol ($H_2O$ saturated).

flux, however, is different depending upon the geometry. For radial flow the expression that is equivalent to eqn. (9) is

$$q = v_f A' = v_f \int_0^w y^2 \, \mathrm{d}w/y \tag{18}$$

In the radial case discussed here, $A'$, rather than an area, is obviously defined by

$$A' = \int_0^w y^2 \, \mathrm{d}w/y \quad \text{(radial flow)} \tag{19}$$

Using $A'$ in place of $A$, eqns. (10–12) are valid for radial flow. Furthermore by defining

$$\mathrm{f}'(y,c) = \frac{A'}{w + c} \tag{20}$$

and letting $z_f$ be the radial distance to the solvent front, eqns. (13–15) are obtained. From eqn. (15) it is possible to generate the zone migration as before. The distance migrated by the spot divided by the distance moved in the same time by the solvent front is defined as $R_R$. The expression for $R_R$ is equivalent to eqn. (16)

$$R_R = \frac{s - s_0}{z_f - s_0} = \frac{S - 1}{Z - 1} \tag{21}$$

and the limiting value of $R_R$ is found as $R_R = y$

$$R_R = \mathrm{f}'(R_R, c) \tag{22}$$

The relationship between limiting $R_R$ and $R_F$ values is of considerable importance in establishing a uniform interpretation of chromatographic data. Unfortunately the $R_R$ of eqn. (22) and the $R_F$ of eqn. (17) are not simply related. This fact is borne out by the experimental results and is illustrated in Fig. 10. Furthermore, the $R_R$ values vary from one another for different wick dimensions. Thus lower $R_R$ values are obtained with long narrow wicks than with short wide ones because of the lesser amount of solvent on the paper. This appears quantitatively in terms of a diminished value for $A'$.

It has often been assumed that the $R_F$ and $R_R$ values are related by

$$R_R^2 = R_F \tag{23}$$

This equation is correct for limiting $R_R$ and $R_F$ values if one can assume a uniform, equal concentration in the two papers. In actual practice it is quite inadequate as will be shown in the experimental section. The equation can be derived by assuming the concentration in both the rectangular and radial cases to be a uniform concentration, $w$, from the source to the solvent front. Making this assumption, the value of $A$, eqn. (9), is $w$. Using this in eqn. (13) we find $f(y,c) = w/(w + c)$. Using eqn. (17) we find also that $R_F = w/(w + c)$. In the radial case we find that $A' = w/y$ when the concentration is assumed uniform. From eqn. (20), $f'(y) = w/y\,(w + c)$. Using eqn. (22) we have

$$R_R = f'(R_R,c) = w/R_R(w + c)$$

or

$$R_R^2 = w/(w + c) \tag{24}$$

Hence eqn. (23) is correct for the profiles assumed above. A uniform profile, however, is a poor approximation to the actual behaviour of paper.

Gravitational and other external fields are not easily analyzed in relationship to zone migration. The principle difficulty lies in the inclusion of a term for flow due to the potential field. This term renders eqn. (2) incorrect, and the Boltzmann transformation, which leads directly to the concept of reduced concentration profiles, is no longer appropriate. The profile can, however, be developed by numerical methods along with the rate of zone migration. This problem is under investigation. It is evident that the concentration profiles for ascending flow eventually lie considerably below those for descending flow, resulting in a similar change in the $R_F$ values.

## EXPERIMENTAL

A small room was thermostated at 30° and saturated with water vapor. The chromatographic chambers were sealed and the bottoms covered with solvent to provide maximum area for saturating the interior atmosphere. Sheets of saturated paper were placed in the chambers to provide additional surface.

The chromatographic paper was dessicated over $P_2O_5$ *in vacuo* for about 24 h prior to use and exposed to solvent vapors prior to chromatographing. Concentration profiles were determined by sectioning the paper and weighing on an analytical balance in stoppered weighing bottles. Dry weights were ascertained after suitable dessication and corrections for adsorbed water were made.

The solvent system was *n*-butanol saturated with water. The paper was Whatman 3 MM chromatographic sheets. The Methyl Orange used for the zone substance was applied with a hypodermic needle as a 1/3 saturated solution in the developing solvent.

The initial spot size was approximately 2 mm in diameter. All chromatograms were developed in the machine direction of the paper by horizontal technique. The wicks used in the semicircular cases were uniform 1 cm in width and of reported lengths.

## RESULTS

From the initially determined reduced concentration profile for linear flow of solvent in paper, a set of $R_F$ *versus* reduced distance of travel, $S$, curves were calculated by a numerical method, using relation (15) for various values of the partition parameter, $c$. This is shown in Fig. 7. These curves show how a chromatographic zone approaches a limiting value asymptotically. As may be expected, this approach is quite rapid for both very high and very low values of $R_F$ but is an important feature with materials of intermediate $R_F$ value.

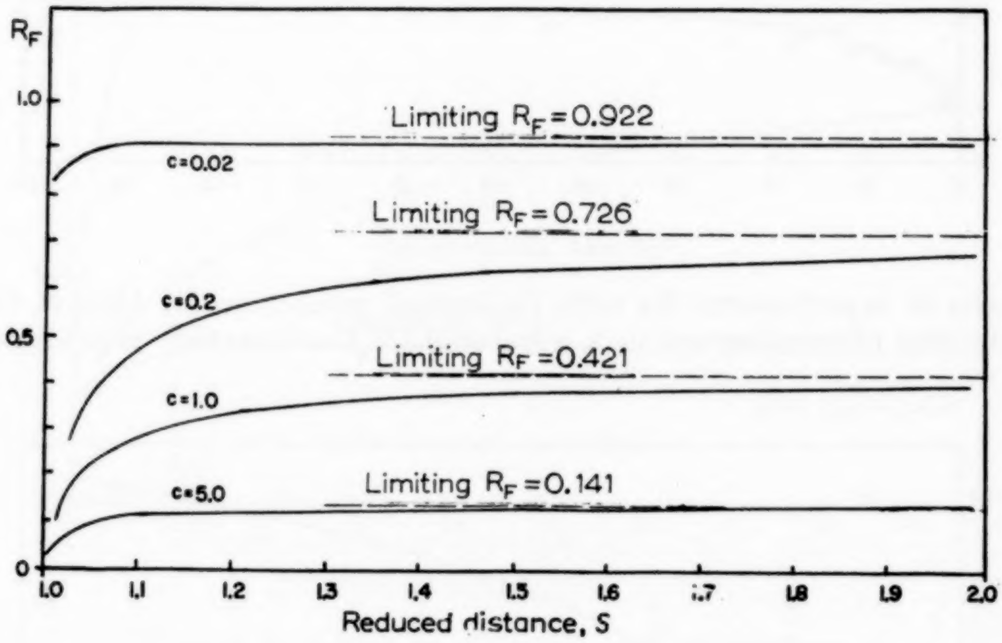

Fig. 7. $R_F$ value as a function of reduced zone travel for various valves of the partition parameter, $c$, for linear, horizontal flow in a $n$-butanol ($H_2O$ saturated) Whatman 3 MM system.

A long developed chromatograph of Methyl Orange was made with a low initial spot placement, $s_0$, to yield an approximation to the limiting $R_F$ value for this substance. From this the partition parameter was estimated to be $c = 1.3$ and the theoretical behavior of $R_F$ as a function of $S$ was calculated.

Methyl Orange was then chromatographed from starting positions of $s_0 = 2, 4, 8, 16$, and 24 cm and the $R_F$ of the zone was observed as a function of the reduced distance. These experimental points are given in Fig. 8 in comparison with the theoretical prediction. The experimental points are in reasonable agreement with the predicted behavior, supporting the proposed theory. The experiments further confirm the expression of this behavior in terms of the reduced distance variable, $S$, which removes the dependence of $R_F$ on initial zone location.

As a further test of the theory presented, it was proposed to predict $R_R$ values for circular flow from the information gained (*i.e.* $c = 1.3$) in the rectangular flow experiment. A wick–semicircle system was used as the concentration profiles could be varied in the semicircle by adjustment of the wick length. The concentration profiles can be obtained numerically from the rectangular profile but for this case it is simpler to determine them experimentally. The radial concentration profiles are given in Fig. 6 for the various wick lengths used. The zero wick length profile was determined on a 20° wedge. The results are shown in Fig. 9.

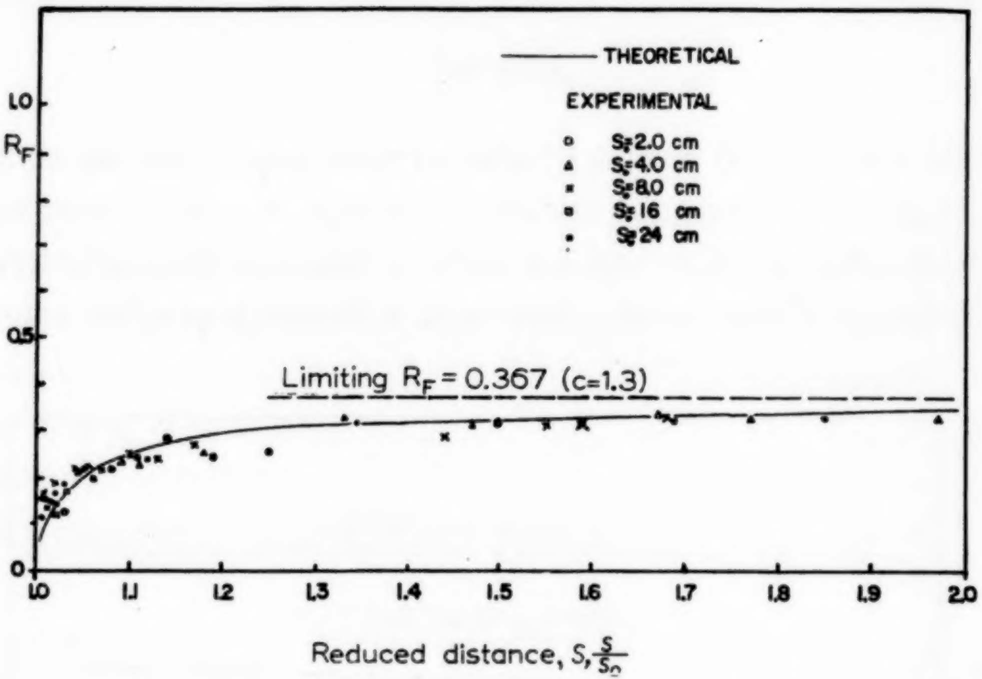

Fig. 8. Comparison of experimental $R_F$ with theoretical prediction for Methyl Orange ($c = 1.3$) on a linear, horizontal chromatogram in a *n*-butanol ($H_2O$ saturated) Whatman 3 MM system.

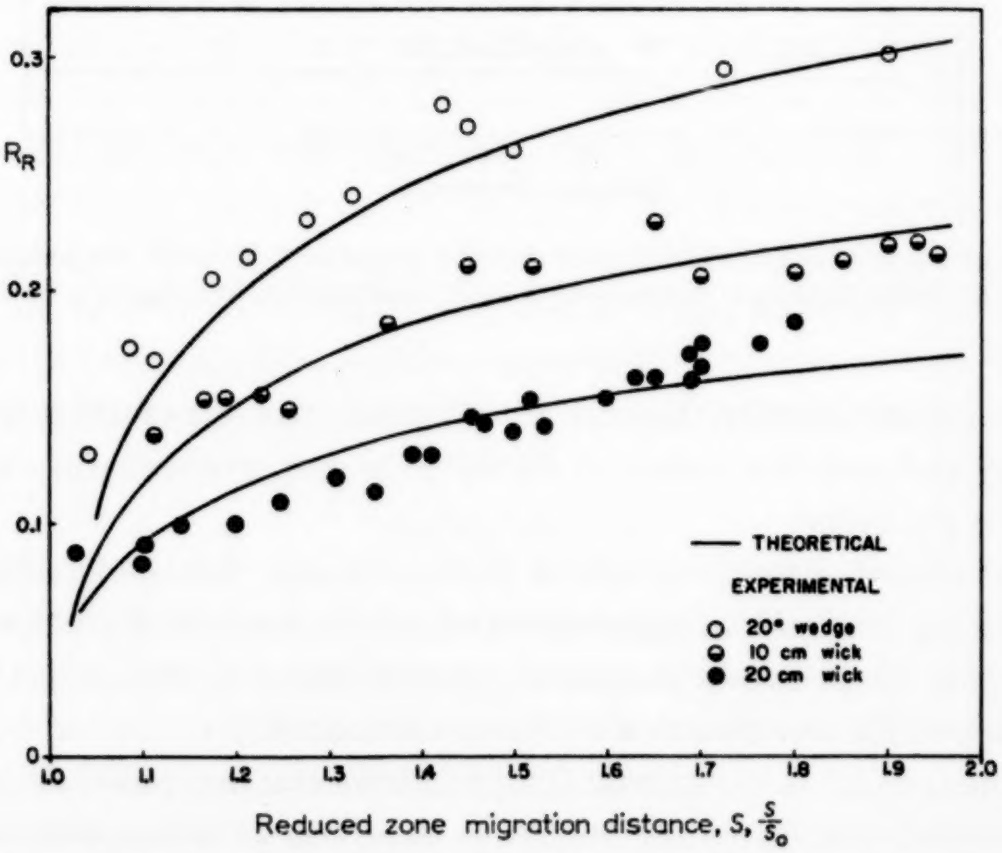

Fig. 9. Experimental and theoretical zone migration for radial cases.

Using the partition coefficient determined in the linear experiment and the concentration profile for the 20 cm wick length, the $R_R$ and $R_R^2$ *versus* $S$ behavior of the zones was predicted as shown in Fig. 10. It is apparent that neither $R_R$ nor $R_R^2$ can be compared directly with rectangular $R_F$'s. Despite the considerable experimental error of the experimental $R_F$ values, the results are quite conclusive in support of the arguments presented here. The curves do not always lie in the order $R_F$, $R_R$, and $R_R^2$, however. Under some circumstances $R_R > R_F$, but whether or not this is true, the $R_R^2$ values are smallest of the three. The parameter which chromatographically characterizes a system is the partition parameter and not the $R_F$ value.

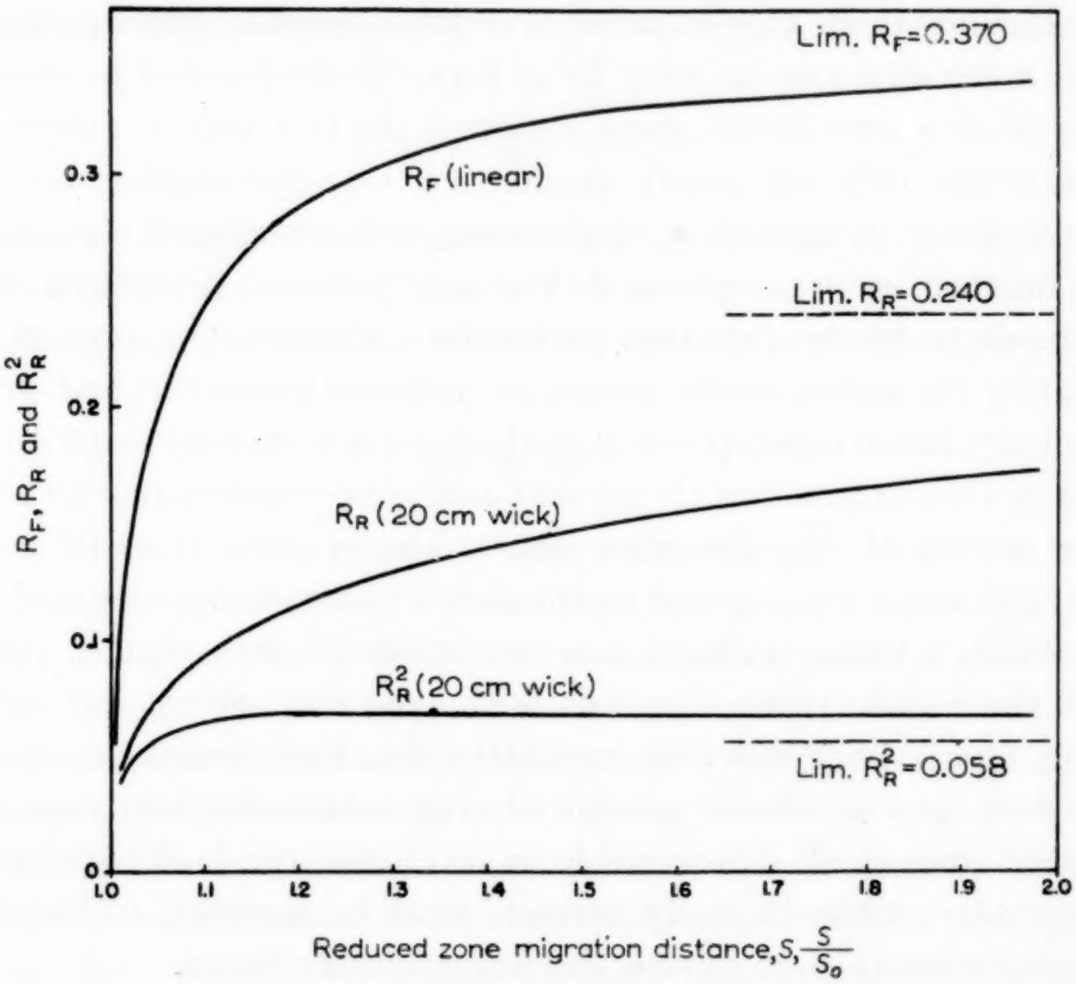

Fig. 10. Comparison of $R_F$, $R_R$ and $R_R^2$ values.

## DISCUSSION AND CONCLUSIONS

The theoretical and experimental results obtained above illustrate several precautions that must be taken in interpreting data in paper chromatography. The role of the concentration gradient is extremely important. Its presence vitiates theories of paper chromatography based upon uniform concentration profiles. Such theories appear deficient in two respects. First, with zones started an appreciable distance from the solvent source, a transient period occurs during which the $R_F$ changes from an initial value of zero to some limiting value. This transition is important, as a rough rule, for a zone migration distance equal to the original distance from the solvent source, $s_0$. This effect has been observed by several workers[1].

More important than the transient unsettled period is the fact that the limiting $R_F$ values are determined by local concentrations. The procedure whereby an average concentration is assumed for all solute zones is incorrect, and does not lead to the proper relationship between the partition coefficient and the $R_F$. Thus in the experimental work reported here, $c$ has to be assigned the value 1.50 rather than 1.30 to arrive at the correct $R_F$ value. Consequently both $c$ and $\alpha$ are in error by about 15 % using the classical method. This situation is rectified by observing the correct limiting $R_F$, and then using eqn. (17) to solve for $c$. Eqn. (5) can be used to obtain $\alpha$ from $c$.

Another point shown in the above results is the fact that no simple comparison exists between the different paper geometries, *i.e.*, rectangular and radial. The relationship of these to ascending, descending and centrifugal chromatography is expected to be even more complicated. The only common denominator of all these methods appears to be the partition parameters, $\alpha$ and $c$. Our present inability to define accurately the nature of the stationary phase in paper chromatography leaves a certain arbitrary factor in independent methods for determining partition coefficients. It is believed by the authors that the present analysis provides a tool for the investigation into the nature of the stationary phase. The problem is under consideration.

Several limitations are imposed on the above treatment by virtue of the assumptions made. First, a linear isotherm has been assumed such that $R$, the fraction of molecules in the mobile phase, depends only upon the solvent but not the solute concentration. Overloading can thus invalidate the above treatment. Also the paper must be free from any significant amount of evaporation. For long runs this problem becomes crucial since small differences from saturation can lead to significant losses. Finally, the theory, while basically correct, must be modified to handle the cases where gradients other than in solvent concentration are found.

## TABLE OF SYMBOLS

| | |
|---|---|
| $\alpha$ | partition coefficient |
| $A$ | area shown in Fig. 3 |
| $A_M$ | cross-sectional area of mobile phase normal to direction of flow |
| $A_S$ | cross-sectional area of immobile phase normal to direction of flow |
| $c$ | partition parameter, $\alpha w A_S/A_M$ |
| $D$ | effective diffusion coefficient for solvent flow in paper |
| $\varkappa$ | flow rate coefficient |
| $q$ | flux |
| $R$ | fraction of molecules in the mobile phase |
| $R_F$ | spot divided by solvent front distance for rectangular flow |
| $R_R$ | spot divided by solvent front distance for radial flow |
| $s$ | distance of zone from bulk solvent |
| $s_0$ | initial zone position |
| $S$ | reduced zone distance, $s/s_0$ |
| $t$ | time |
| $\bar{u}$ | mean velocity of the solute zone |
| $v$ | average local solvent velocity |
| $v_f$ | velocity of the solvent front |

$w$ concentration, (g of solvent)/(g of dry paper)
$y$ reduced distance, $z/z_f$
$z$ distance from bulk solvent
$z_f$ distance of solvent front from bulk solvent
$Z$ reduced solvent front distance, $z_f/s_0$

## ACKNOWLEDGEMENT

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## SUMMARY

The influence of concentration gradients upon zone migration has been investigated theoretically and experimentally. It is shown that the equation $R_F = A_M/(\alpha A_S + A_M)$. is incorrect in relating the $R_F$ to the partition coefficient $\alpha$. The $R_F$ shows an initial unsettled period during which it approaches a limiting $R_F$ value. Both the limiting and transient values were calculated for the migration of Methyl Orange on Whatman 3 MM paper with a water-saturated butanol solvent.

Cases of radial flow with variable length wicks were also treated. Using the data from rectangular flow, $R_R$ values were successfully predicted for the same Methyl Orange system. The reasons for deviation from the relationship $R_R^2 = R_F$ were discussed.

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# A SIMPLE METHOD OF PLOTTING THEORETICAL CURVES OF $R_F$ AS A FUNCTION OF pH IN BUFFERED PAPER CHROMATOGRAPHY

ANDRZEJ WAKSMUNDZKI AND EDWARD SOCZEWIŃSKI
*Medical Academy, Lublin (Poland)*

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In buffered paper chromatography of organic electrolytes it is often possible to obtain optimal separation conditions by changing the partition ratios of the substances between both phases (and thus the rate of migration) by the use of buffer solutions as the stationary phase[1]. The $R_F$–pH relationship is then expressed by the formula[2]:

$$R_F = \frac{kr}{kr + 1 + \frac{K_A}{[\mathrm{H}^+]}} \text{ (acid)} \quad \text{or} \quad R_F = \frac{kr}{kr + 1 + \frac{K_B}{[\mathrm{OH}^-]}} \text{ (base)}$$

where $k$ is the partition coefficient of unionized substance between the mobile and immobile phase, $r$ is the ratio of cross-sectional areas of mobile and immobile phases ($kr$ is thus the partition number), and $K_A$ and $K_B$ are ionization constants.

When $R_F$ measurements are interpreted, these functions should be plotted. Determinations of parameters involved ($k$, $r$, $K_A$) and calculation and plotting of the curves takes a lot of time. Particularly when there are many determinations to be interpreted much time and work can be saved by taking advantage of a property of the function $R_F = \mathrm{f(pH)}$. If the ionization constant $K_A$ is increased $x$ times, it is sufficient to increase the hydrogen ion concentration $x$ times (or to decrease the pH by log $x$ units) for the $R_F$ value to remain unchanged. This is valid for the whole curve; thus the ionization constant does not influence the shape of the curve but only its position: an $x$-fold increase of $K_A$ shifts the curve, without changing its shape, by log $x$ pH units towards lower pH values.

$R_F = \mathrm{f(pH)}$ curves of organic bases are mirror reflections of curves of acids and an $x$-fold increase of $K_B$ shifts these curves, unchanged, by log $x$ pH units towards higher pH values.

This property permits the use of a simple method of plotting $R_F = \mathrm{f\ (pH)}$ curves (see Fig. 1).

Fig. 1, representing plots of $R_F = \mathrm{f\ (pH)}$ curves for acids, at various $kr$ values (partition number of the unionized substance), is copied onto a transparent sheet. On a second sheet identical coordinates are drawn. The second sheet is laid upon the first sheet and then shifted horizontally, with pH axes overlapping, until the point marked X on the first sheet coincides with the pH value equal to the p$K_A$ of the acid under consideration. Then the curve with respective *kr* value is copied. If the substance considered is a base, the first sheet (with Fig. 1) is turned over, curves for bases being mirror reflections of curves for acids. Point X must then coincide with a pH value on the second sheet equal to 14—p$K_B$.

Working backwards, when theoretical curves from Fig. 1 are made to fit points found experimentally, for an acid of a known partition number *kr*, the point X will indicate a pH value on the pH coordinate of the experimental plot equal to the p$K_A$ value of the acid (or 14 — p$K_B$ value if the substance is a base).

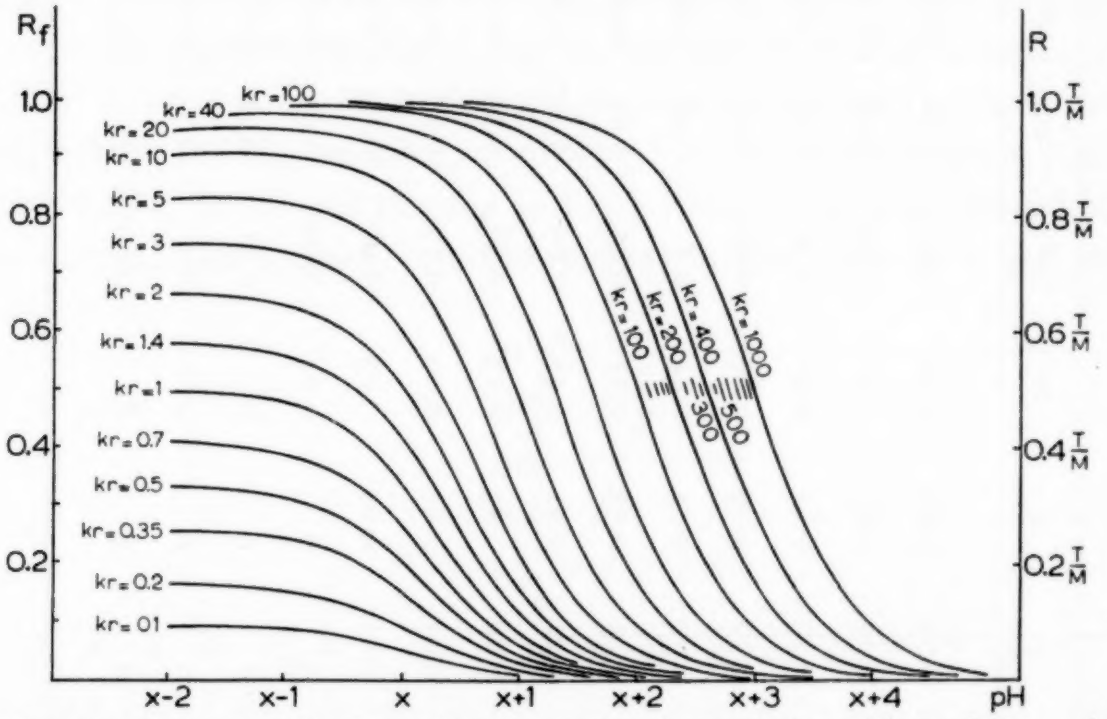

Fig. 1. The influence of the partition number *kr* on the shape and position of $R_F$ = f (pH) curves.

Comparison of the theoretical and experimental curves may give information concerning the mechanism of chromatography and make it possible to find ionization constants of acids and bases and isoelectric points of ampholytes. Moreover, if the experimental $R_F$ = f (pH) curve is in accordance with theory (which holds if paper chromatography is a continuous extraction process in the strict sense of the word) only very few $R_F$ values found experimentally at various pH values are needed to predict the whole curve. In fact, for an acid or base only two such points are sufficient to allow one to plot the whole curve: one at the pH in which the substance shows its maximum $R_F$ value (for a given solvent and humidity of the paper), the second at the pH at which the substance has an intermediate $R_F$ value (0.2–0.8 $R_F$ max.). For an ampholyte three $R_F$ values are needed: (1) at the pH at which the substance shows its maximum $R_F$ value, (2) at the pH at which the substance has an intermediate $R_F$ value owing to acidic ionization, and (3) at the pH at which the substance has an intermediate $R_F$ owing to basic ionization.

*Examples*

(a) For an organic base it was found that at pH 6 the $R_F$ is 0.83. This is its highest $R_F$ since at pH 7 also the $R_F$ is 0.83. At pH 3 the $R_F$ was found to be 0.42. After turning over Fig. 1 (since the substance is a base) we find that only one curve can be

TABLE I

| *pH* \ *kr* | *0.1* | *0.2* | *0.35* | *0.5* | *0.7* | *1* | *1.4* | *2* | *3* | *5* | *10* | *20* | *40* | *100* | *200* | *400* | *1000* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| X — 2 | 0.09 | 0.17 | 0.26 | 0.33 | 0.41 | 0.50 | 0.58 | 0.67 | 0.75 | 0.83 | 0.91 | 0.95 | 0.98 | 0.99 | 1.0 | 1.0 | 1.0 |
| X — 1.5 | 0.09 | 0.16 | 0.25 | 0.33 | 0.41 | 0.49 | 0.58 | 0.66 | 0.75 | 0.83 | 0.91 | 0.95 | 0.98 | 0.99 | 1.0 | 1.0 | 1.0 |
| X — 1 | 0.08 | 0.15 | 0.24 | 0.31 | 0.39 | 0.48 | 0.56 | 0.65 | 0.73 | 0.82 | 0.90 | 0.95 | 0.97 | 0.99 | 1.0 | 1.0 | 1.0 |
| X — 0.5 | 0.07 | 0.13 | 0.21 | 0.28 | 0.35 | 0.43 | 0.52 | 0.60 | 0.70 | 0.79 | 0.89 | 0.94 | 0.97 | 0.99 | 1.0 | 1.0 | 1.0 |
| X | 0.05 | 0.09 | 0.15 | 0.20 | 0.26 | 0.33 | 0.41 | 0.50 | 0.60 | 0.71 | 0.83 | 0.91 | 0.95 | 0.98 | 0.99 | 1.0 | 1.0 |
| X + 0.5 | 0.02 | 0.05 | 0.08 | 0.11 | 0.14 | 0.19 | 0.25 | 0.32 | 0.42 | 0.55 | 0.71 | 0.83 | 0.91 | 0.97 | 0.98 | 0.99 | 1.0 |
| X + 1 | 0.01 | 0.02 | 0.03 | 0.04 | 0.06 | 0.08 | 0.11 | 0.15 | 0.21 | 0.31 | 0.48 | 0.65 | 0.79 | 0.90 | 0.95 | 0.98 | 0.99 |
| X + 1.5 | 0 | 0.01 | 0.01 | 0.02 | 0.02 | 0.03 | 0.04 | 0.06 | 0.08 | 0.13 | 0.23 | 0.38 | 0.55 | 0.76 | 0.86 | 0.93 | 0.96 |
| X + 2 | 0 | 0 | 0 | 0 | 0.01 | 0.01 | 0.01 | 0.02 | 0.03 | 0.05 | 0.09 | 0.17 | 0.28 | 0.50 | 0.67 | 0.80 | 0.91 |
| X + 2.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.01 | 0.01 | 0.02 | 0.03 | 0.06 | 0.11 | 0.24 | 0.39 | 0.56 | 0.76 |
| X + 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.01 | 0.02 | 0.04 | 0.09 | 0.17 | 0.29 | 0.50 |
| X + 3.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.01 | 0.01 | 0.03 | 0.06 | 0.12 | 0.24 |
| X + 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.01 | 0.02 | 0.04 | 0.09 |
| X + 4.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.01 | 0.01 | 0.03 |

made to fit these experimental points: the $kr = 5$ curve (Fig. 2). Point X of Fig. 1 is situated at pH 3.7 on the experimental plot; so $pK_B = 14 - 3.7 = 10.3$; $K_B = 5 \cdot 10^{-11}$.

(b) For an ampholyte it was found that $R_F = 1$ at pH 7, $R_F = 0.5$ at pH 11 (acidic ionization) and $R_F = 0.4$ at pH 1 (basic ionization). The partition number of the substance is here above 50 and it is impossible to estimate it from $R_F$ max. because $kr$ values above 50 no longer influence the shape of the curve, only its position. So we may copy onto the experimental plot any of the curves of Fig. 1 whose $kr > 50$, for instance the $kr = 100$ curve. At higher pH we copy the curve in its normal position (the ampholyte behaves like an acid), at lower pH in reversed position (the ampholyte behaves like a base). So we get the $R_F$ = f (pH) relationship in the whole pH range (for a given solvent and humidity of the paper) in the form of a bell-shaped curve (Fig. 3). The isoelectric point of the ampholyte lies on the axis of symmetry of the curve. It is impossible, however, to estimate ionization constants of the ampholyte since we do not know its partition number $kr$; to find this other experiments will be necessary. For instance, if we find that $kr = 100$, point X of Fig. 1 indicates $pK_A = 9.0$; $pK_B = 14 - 3.2 = 10.8$; when $kr = 500$ we find that $pK_A = 8.3$; $pK_B = 14 - 3.9 = 10.1$. For plotting theoretical curves in any scale, Table I may also be used. The method described is also applicable to partition chromatography on buffered columns. Ordinate values must then be multiplied by $T/M$ ($T$ is the total cross-sectional area of column, $M$ is the cross-sectional area of the mobile phase), and the $R$ value used in column chromatography is obtained instead of $R_F$ (see coordinates on the right-hand side of Fig. 1). The method described has been confirmed experimentally for quinoline derivatives.

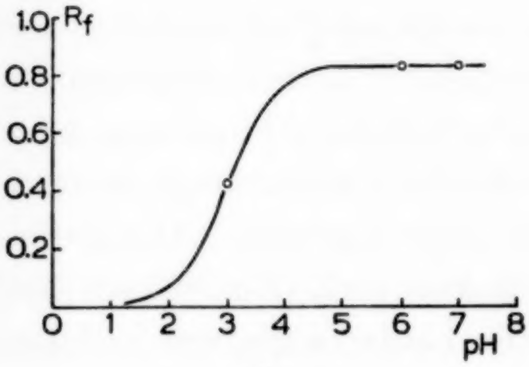

Fig. 2.

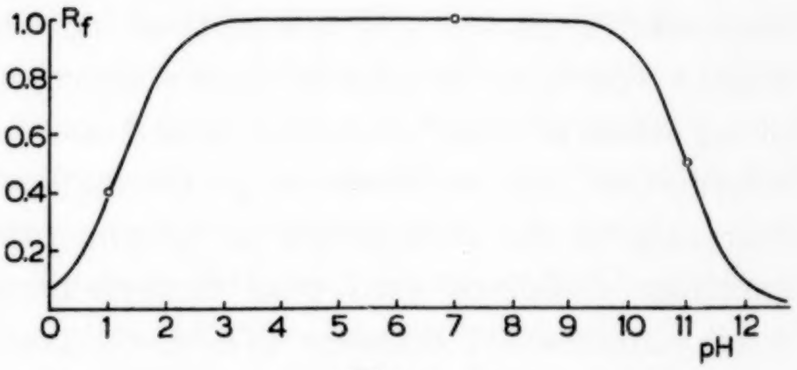

Fig. 3.

## SUMMARY

A simple method of drawing theoretical $R_F$ = f (pH) curves of organic electrolytes is described.

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# USE OF DEAE-CELLULOSE IN THE SEPARATION OF PROTEINS FROM EGG WHITE AND OTHER BIOLOGICAL MATERIALS

STANLEY MANDELES

*Western Regional Research Laboratory**, *Albany, Calif.* (*U.S.A.*)

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Cellulose ion-exchange chromatography has emerged recently as a powerful tool for separation and identification of proteins in simple and complex biological mixtures. Successful application of this method has been made in the analysis of horse and human sera[1, 2], purification of various enzymes and hormones[3–7], and characterization of egg white proteins[8]. Since the appearance of these early reports, a large amount of information has accumulated on the application of cellulose ion-exchange chromatography to the purification of specific proteins[9]. In most instances, the anion-exchange celluloses used were diethylaminoethyl cellulose (DEAE-cellulose) and triethylaminoethyl cellulose (TEAE-cellulose), and the cation exchange cellulose used was carboxymethyl cellulose (CM-cellulose).

During the course of an investigation of the protein constituents of chicken egg white and yolk, we have employed both DEAE-cellulose and CM-cellulose. The former was more useful in separating the components of fresh unaltered egg white because elution could be largely accomplished near neutrality at low ionic strength. We have also found that the proteins of other complex biological materials of either plant, animal, or bacterial origin may be separated by DEAE-cellulose chromatography, although for egg yolk, a few preliminary purification steps are necessary. Our experiences with DEAE-cellulose in the separation of proteins from such mixtures will be described in this report.

## MATERIALS AND METHODS

DEAE-cellulose, type 20 (coarse grade), 0.7 mequiv./g, was purchased from Brown Company, Berlin, New Hampshire. Before use, the DEAE-cellulose was washed on a Büchner funnel with fritted glass disc successively with 0.1 *N* HCl–1 *M* NaCl, water, 1 *M* $NaHCO_3$, water, 1 *M* $Na_2CO_3$, water, 0.1 *N* NaOH, water, ethanol and water. The cellulose was then suspended in water overnight to permit trapped air bubbles to escape. After the suspension had settled, any fines which remained were decanted.

### *Preparation of columns*

The washed cellulose was suspended in 0.1 *N* NaOH and portions were poured into glass chromatography columns of appropriate size for the intended use, usually, 2.5 cm diameter and 40 cm long. When the cellulose had settled to the desired height, further additions were discontinued and the column was washed to neutrality with water. This was followed by a wash with 0.02 *M* glycine until a blue band, which appears to be a Schiff's base, was washed out of the column. The symmetry of this blue band as it migrated down the column served as an effective indicator of the levelness of packing of the column.

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* A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

### *Elution schedules*

The majority of the separations described below were carried out with a non-linear gradient elution schedule adapted from SPEERS *et al.*[2]. With egg white, for example, 5 ml of carefully blended[10] material were mixed with an equal volume of 0.02 *M* glycine and the mixture was entered on a 2.5 × 40 cm column. The diluted egg white was then permitted to sink almost entirely into the column before being washed with two 10-ml portions of glycine solution. Gradient elution was then begun with 750 ml of 0.02 *M* glycine in the mixing flask and a reservoir containing 0.02 mole each of $KH_2PO_4$, $K_2HPO_4$, and glycine in 1 l of solution. The flow rate was adjusted to 2 ml/min. When the solution in the reservoir was exhausted, it was replaced by 1 l of solution containing 0.1 mole $KH_2PO_4$, 0.1 mole NaCl, and 0.02 mole glycine. After this volume was used, the solution was replenished with 1 l of solution containing 0.1 mole $KH_2PO_4$, 0.1 mole NaCl, 0.03 mole HCl, and 0.02 mole glycine.

In the chromatography of egg white for preparative purposes, the white of an entire egg was carefully blended, diluted with an equal volume of 0.02 *M* glycine, entered on a column 5.0 cm in diameter and 60 cm long, and followed by two washes with equal volumes of glycine solution. Elution of the egg white protein was accomplished by changing the composition of glycine–phosphate buffer added to the column in discrete steps.

The course of the elution of proteins was followed by measuring the optical density of the fractions collected at 280 m$\mu$. It was found, however, that modification of the elution schedule could be assessed more easily when the eluant was monitored by a UV absorption monitor and recording meter*. In this manner, a continuous graphic record of the absorption at 254 m$\mu$ of the emerging solutions was obtained without necessity of collecting and analyzing individual fractions.

### *Assays for egg white proteins*

In experiments with egg white, ovalbumin was assayed for content of free SH groups by the method of BOYER[11]; lysozyme was identified and estimated by its lytic activity on *Micrococcus lysodeikticus*[12]; conalbumin was measured by its iron binding capacity[13]; and ovomucoid was detected by its antitrypsin activity[14]. The biotin-binding capacity of avidin was measured by the HERTZ modification of the EAKIN method[15].

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* Gilson Medical Electronics, Middleton, Wisconsin. Mention of specific products does not constitute endorsement by the Department of Agriculture.

### *Muscle extract*

Chicken breast muscle extract was prepared by blending freshly excised chicken breast muscle with an equal weight of cold distilled water in a Waring Blendor. The homogenate was centrifuged at 15,000 *g*, and the supernatant was dialyzed overnight against running distilled water. After dialysis, the supernatant was clarified by centrifugation and chromatographed. Assays were made for phosphorylase[16], phosphoglucose isomerase[17], and aldolase[18]. Adenylate kinase[19] was detected by following the disappearance of ADP and the concomitant appearance of ATP and AMP on paper chromatograms.

### *Cabbage extract*

Cabbage extract was prepared by blending fresh cabbage with an equal volume of 0.5 *M* sucrose. The homogenate was centrifuged at 23,000 *g*, and the supernatant was examined chromatographically with no prior dialysis. Assays[20] were made for apyrase, inorganic pyrophosphatase and acid phosphatase activity*.

### *Bacterial extracts*

*Escherichia coli* ATC 11246 and *E. coli* 6-204-55** were grown under identical conditions to approximately the same turbidity in a 3% trypticase-soy broth. Cells from each of the cultures were harvested, washed with water, and disrupted by ultrasonic vibration. The sonicate was clarified by centrifugation at 23,000 *g* and dialyzed against distilled water overnight in order to remove low molecular weight UV-absorbing material. After dialysis, the sonicates were examined successively with two columns of DEAE-cellulose, 1 cm diameter and 30 cm long, which had been made at the same time. The fractions obtained from the chromatographic analysis of *E. coli* ATC 11246 were assayed for presence of L-glutamic acid decarboxylase[21].

## RESULTS

### *Egg white*

When 5 ml of fresh egg white containing approximately 600 mg of protein were chromatographed on DEAE-cellulose according to the gradient elution schedule described above, the pattern shown in Fig. 1 was obtained. One tube from each peak was assayed for lysozyme, conalbumin, ovalbumin, and ovomucoid. Lysozyme activity was found in peaks A, B and C, conalbumin in peak D, ovalbumin in peaks G and H, and after dialysis, ovomucoid activity in peak E. The flavoprotein component previously described by RHODES *et al.*[8] was identified with peak J. Although not discernible as a distinct peak, avidin activity was found in fractions 52 and 53. Peaks F, I and K were not characterized.

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* Cabbage extract as well as the apyrase and phosphatase assays were generously provided by Dr. M. MAZELIS of this Laboratory.

** *E. coli* 6-204-55 was obtained from the laboratories of Dr. MAX LEVINE, Territorial Dept. of Health, Honolulu, Hawaii, and is reported to contain the antibiotic colicin.

An ultraviolet absorption spectrum was determined for peaks A, D and H, and the results are shown in Fig. 2a. For purposes of comparison, the ultraviolet absorption spectra of crystalline lysozyme, conalbumin and ovalbumin are shown in Fig. 2b. It can be seen that the spectra of peaks A, D and H are indistinguishable from those of the corresponding purified crystalline proteins. In addition, it was found that the ultraviolet absorption spectra of peaks B and C were also similar to that of lysozyme. Whether these components represent a molecular species separate from the main lysozyme or the result of lysozyme interacting with other proteins or the cellulose is not known at present. Some preliminary evidence indicates that peaks B and C have a 20 % higher specific activity than ordinary lysozyme, and in addition are more easily lost during dialysis. Similarly, a comparison of the ultraviolet absorption spectra of peaks G and H shows them to be indistinguishable. It can be seen from the pattern in Fig. 1 that separation of individual peaks is not complete. We have found that by judicious selection of the eluting buffers, complete separations can be effected, for example: elution with 0.02 *M* glycine will separate peak A from B and C completely; 0.01 *M* phosphate pH 6.8 will separate peaks B and C from D; and 0.02 *M* phosphate pH 6.8 will separate peak D from peak E, and so on. Considerably longer periods of time are required to achieve complete resolution in this manner, however. Recovery of protein absorbed on the column was determined by submitting material from each of a number of peaks obtained previously by chromatography to an additional chromatographic separation and comparing the optical densities at 283 m$\mu$ and pH 6 before and after rechromatography. Results are shown in Table I. It was found that each of the components tested emerged as a single peak at the same position as the original material, and that only lysozyme and ovomucoid were not

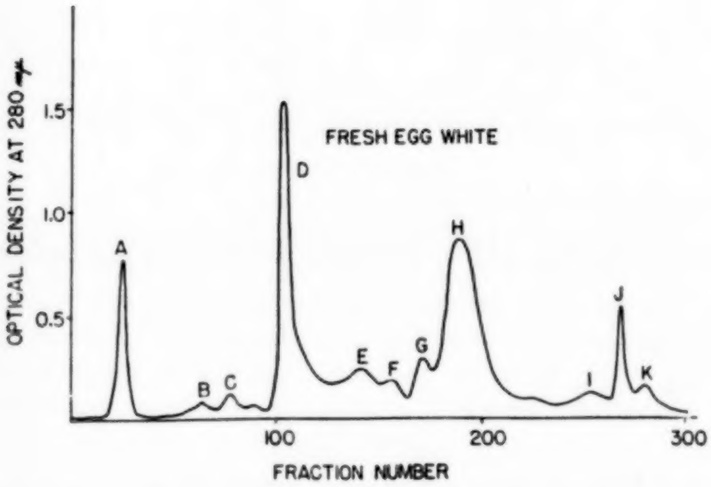

Fig. 1. Chromatography of egg white. Column size: 2.5 cm diameter, 40 cm long. Mixing volume: 750 ml 0.02 *M* glycine. Reservoir: 1 l solution containing 0.02 mole of glycine, 0.02 mole of $K_2HPO_4$, and 0.02 mole of $KH_2PO_4$. Reservoir changed at fraction No. 100 to 1 l solution containing 0.02 mole of glycine, 0.1 mole of $KH_2PO_4$, 0.1 mole of NaCl, and 0.03 mole of HCl. Volume of each fraction: 10 ml.

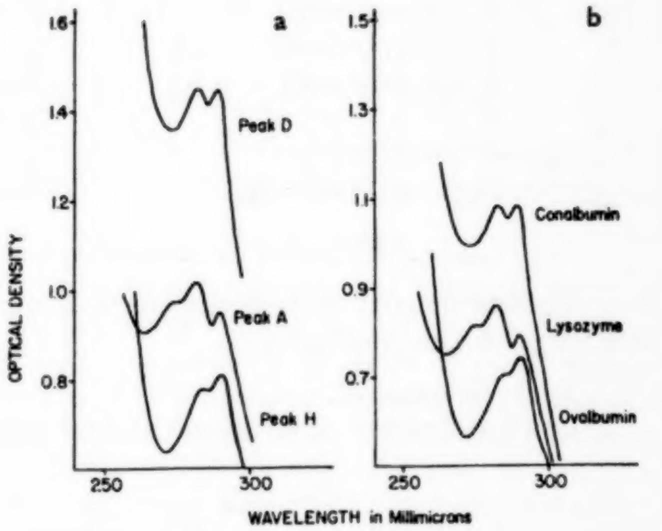

Fig. 2. Ultraviolet spectra of egg white proteins. Spectra of proteins determined in Cary Recording Spectrophotometer. Protein solutions adjusted to pH 13 with 1 *M* NaOH. Proteins in Fig. 2b were prepared in classical manner and recrystallized several times. No purification of proteins in Fig. 2a was attempted.

TABLE I

PERCENT RECOVERY OF PROTEINS

| Peak | *O.D./ml × total volume* before rechromatography | *O.D./ml × total volume* after rechromatography | % Recovery |
|---|---|---|---|
| A* (lysozyme) | 5.86 | 4.45 | 76 |
| D (conalbumin) | 13.24 | 13.20 | 99 |
| E (ovomucoid) | 8.2 | 6.9 | 85 |
| F** | 14.9 | 14.8 | 99 |
| G (ovalbumin-2) | 2.43 | 2.36 | 97 |
| H (ovalbumin-1) | 8.60 | 8.15 | 95 |

Optical density of each sample was measured at 283 mμ, pH 6. Samples were dialyzed before and after chromatography.

* Dialysis omitted.

** Optical density determined at 265 mμ, pH 6.5.

recovered quantitatively. We were unable to detect the presence of possible breakdown products of lysozyme anywhere else in the chromatographic pattern.

### *Reproducibility*

The patterns obtained by gradient elution were reproducible to within 5 % provided that the columns used in the comparison were made at the same time from the same batch of washed DEAE-cellulose, and that the material being chromatographed was the same for each column. Variations in the position of peaks under these conditions could be attributed to differences in flow rate, buffer concentration, and temperature. These differences could be minimized, however, by operating comparative columns simultaneously. We have found that patterns obtained with egg white on columns operated in this manner are practically indistinguishable from each other.

### *Separation of proteins in chicken breast muscle extract*

When 30 ml of chicken muscle extract, containing approximately 10 mg protein/ml were chromatographed on DEAE-cellulose, the pattern shown in Fig. 3 was obtained. The optical densities of only the first 100 fractions are shown in this figure because no additional peaks were eluted beyond this point when the analysis was continued in the same manner as described above for egg white. However, when the pH of the eluting fluid was lowered to 2, fractions consisting mainly of nucleic acid appeared. These fractions were not characterized any further.

Assays for phosphorylase, phosphoglucose isomerase, aldolase, and adenylate kinase were performed on one tube from each of the seven peaks lettered in Fig. 3. Although each of the above enzyme activities was present in the original extract, only adenylate kinase activity could be found after chromatography. This enzyme appeared to be localized wholly within peak F of Fig. 3. In addition, peak A was identified by spectrophotometric examination as cytochrome *c*.

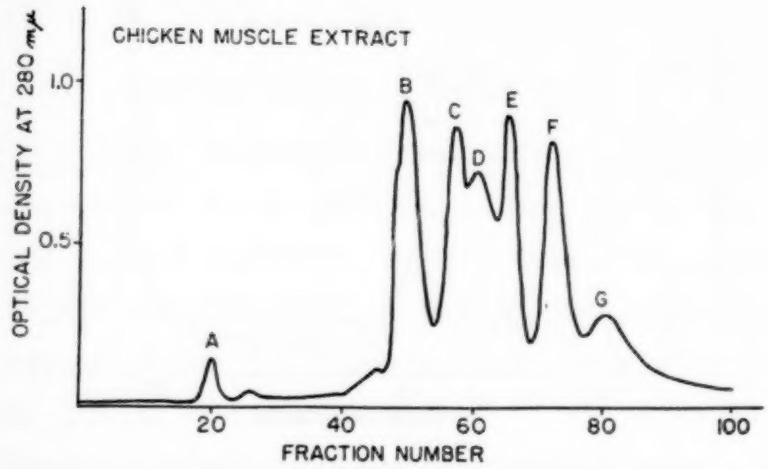

Fig. 3. Chromatography of chicken breast muscle extract. Column size: 2.5 cm diameter, 40 cm long. Mixing volume: 750 ml of 0.02 *M* glycine. Reservoir: 1 l solution containing 0.02 mole of glycine, 0.02 mole of $K_2HPO_4$ and 0.02 mole of $KH_2PO_4$. Volume of each fraction: 10 ml.

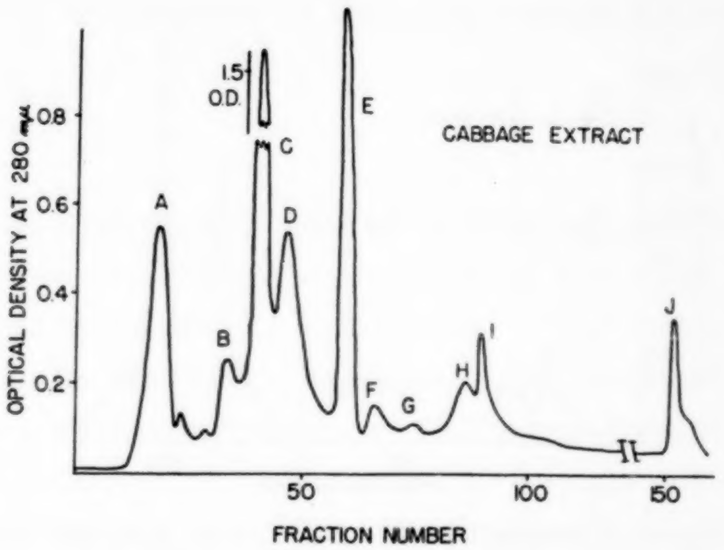

Fig. 4. Chromatography of cabbage extract. Column size: 2.5 cm diameter, 40 cm long. Mixing volume: 750 ml 0.02 *M* glycine. Reservoir: 1 l solution containing 0.02 mole of glycine, 0.02 mole of $K_2HPO_4$, and 0.02 mole of $KH_2PO_4$. Reservoir changed at fraction No. 100 to 1 l solution containing 0.02 mole of glycine, 0.1 mole of $KH_2PO_4$ and 0.1 mole of NaCl. Volume of each fraction: 10 ml.

### *Chromatography of cabbage extract*

Forty ml of undialyzed cabbage extract, containing approximately 10 mg protein/ml were chromatographed and the pattern obtained is shown in Fig. 4. One tube from each of the nine peaks lettered in Fig. 4 was assayed for apyrase, inorganic pyrophosphatase and acid phosphatase activities. Only peak E showed any significant activity, and it contained all three activities. In addition, peak E was characterized by an intense yellow color which, upon spectrophotometric examination at various pH's revealed a spectrum reminiscent of pyridoxal phosphate. Experiments are now in progress to characterize this component.

### *Chromatography of two strains of* E. coli

In some early trials, an attempt was made to isolate L-glutamic acid decarboxylase from extracts of *E. coli* ATC 11246[21] and L-lysine decarboxylase from extracts of *Bacterium cadaveris*[22] by chromatography on DEAE-cellulose. The results of these attempts were largely negative. It was noted, however, that the chromatography patterns of these two organisms were strikingly different. An attempt was made, therefore, to compare the chromatographic patterns of different strains of the same organism that grow at approximately the same rate in a complex medium. Accordingly, cultures of *E. coli* ATC 11246 and *E. coli* 6-204-55 were grown and extracts prepared as described above. The samples used in the chromatography contained 100 mg of protein each, and were chromatographed on columns 1 cm diameter and 30 cm long. The volumes of buffer used in the elution schedule were altered to compensate for these smaller capacity columns. It may be seen that the pattern obtained from *E. coli* ATC 11246 in Fig. 5a is similar to the pattern obtained from *E. coli* 6-204-55 in Fig. 5b in only a few respects, namely the fractions obtained near fraction

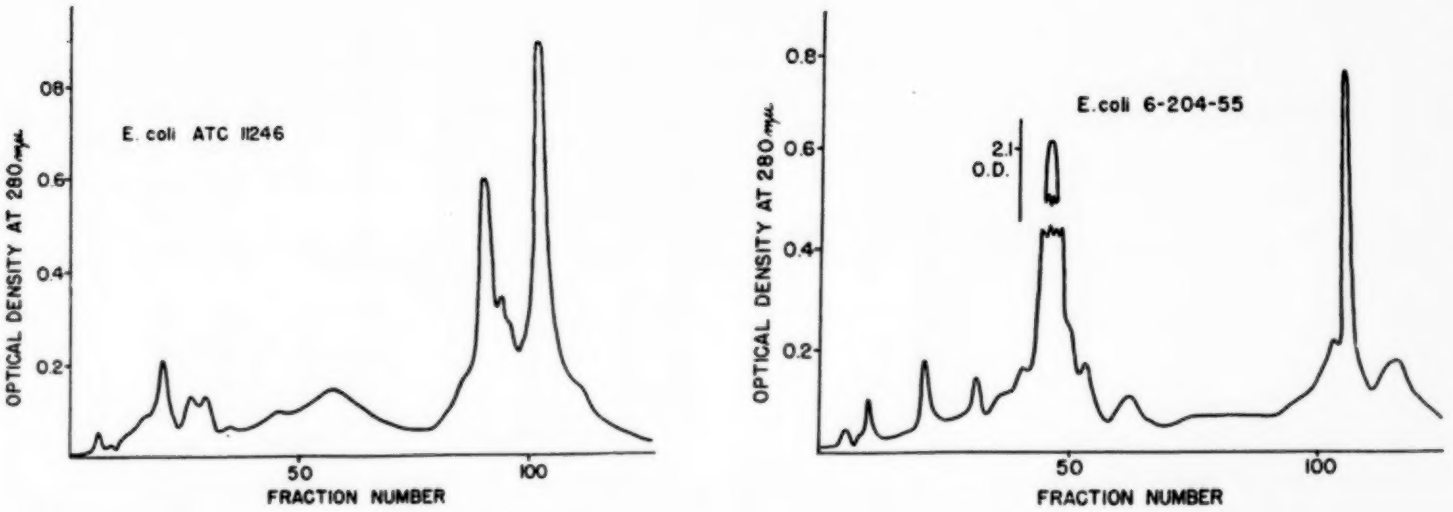

Fig. 5. Chromatography of *E. coli*. Column size: 1.0 cm diameter, 30 cm long. Mixing volume: 400 ml 0.02 *M* glycine. Reservoir: 250 ml solution containing 0.005 mole of glycine, 0.005 mole of $K_2HPO_4$, and 0.005 mole of $KH_2PO_4$. Reservoir changed at fraction No. 50 to 250 ml solution containing 0.005 mole of glycine, 0.025 mole of $KH_2PO_4$ and 0.025 mole of NaCl. At fraction No. 100, reservoir changed to 250 ml solution containing 0.005 mole of glycine, 0.025 mole of $KH_2PO_4$, 0.025 mole of NaCl, and 0.01 mole of HCl. Volume of each fraction: 5 ml.

6, 20 and 105. Other areas of similarity may exist but they are obscured by the components that are obviously different chromatographically.

*Chromatography of the livetin fraction of egg yolk*

After some preliminary trials with whole egg yolk, it became clear that an initial purification was required in order to remove the bulk of the lipid. Accordingly, a separation was made of whole egg yolk into three protein fractions by the method of MARTIN *et al.*[23]. The water-soluble or livetin fraction was chromatographed on DEAE-cellulose, and the pattern shown in Fig. 6 was obtained. A more acidic gradient elution schedule was used for the chromatography of the livetin fraction so that the major portion of the proteins could be eluted in a convenient time period. It can be seen that, as in the case with egg white, the number of components that can be separated by DEAE-cellulose chromatography is greater than the number that can be

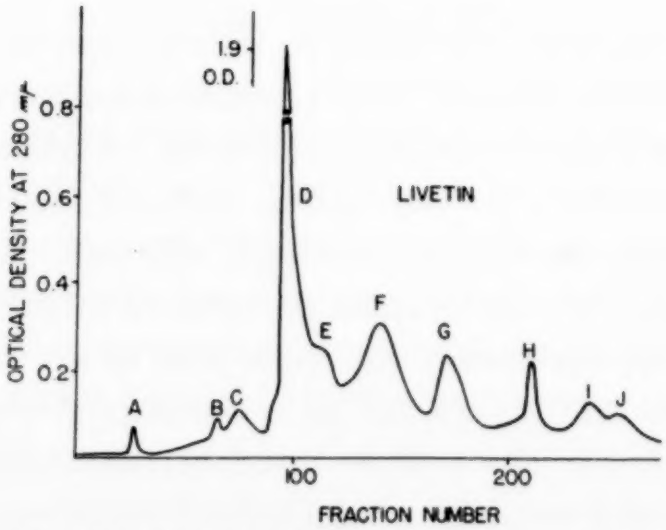

Fig. 6. Chromatography of livetin. Column size: 2.5 cm diameter, 40 cm long. Mixing volume: 750 ml 0.02 *M* glycine. Reservoir: 1 l solution containing 0.02 mole of glycine, 0.05 mole of $KH_2PO_4$, 0.05 mole of NaCl. Reservoir changed at fraction No. 100 to 1 l solution of 0.02 mole of glycine, 0.1 mole of $KH_2PO_4$, 0.1 mole of NaCl. At fraction No. 200, reservoir changed to 1 l solution containing 0.02 mole of glycine, 0.1 mole of $KH_2PO_4$, 0.1 mole of NaCl, and 0.03 mole of HCl. Volume of each fraction: 10 ml.

visualized by electrophoresis[24,25]. Work is now in progress on the characterization of these proteins.

## DISCUSSION

The early electrophoretic studies of LONGSWORTH *et al.*[24] indicated that egg white contained seven protein components. We have found that separation of egg white by DEAE-cellulose chromatography yielded at least 12 components that were easily visualized in the chromatographic patterns. Similarly, SHEPARD AND HOTTLE[25] and MARTIN, VANDEGAER AND COOK[23] found four components in the water-soluble or livetin fraction of yolk by electrophoretic analysis. In our preliminary trials with this material, at least 9 distinct components were found by DEAE-cellylose chromatography. Because of its high resolving power, it seems clear that DEAE-cellulose chromatography is a useful analytical adjunct.

With respect to the effectiveness of DEAE-cellulose chromatography in the separation of enzymes from crude mixtures, our attempts were partially successful in that only one of the 4 enzymes assayed for in muscle extract was found after chromatography, and the assays for L-glutamic acid decarboxylase in extracts of *E. coli* were negative in all the chromatographic fractions tested. It must be pointed out, however, that only the maximum tube from each peak was tested for enzyme activity in these assays. It is quite possible that an enzyme that represents a small fraction of the total protein in a crude biological mixture may be eluted at a point which does not correspond to a peak on the chromatographic patterns. An enzyme eluted in this manner would have been overlooked, therefore, in the selection of samples for assay. It would appear that this difficulty might be overcome by assaying all of the fractions collected.

The observation that two strains of *E. coli*, when chromatographed, yielded different patterns suggests a taxonomic use for DEAE-cellulose chromatography. One might even speculate on its usefulness in genetic investigations where a mutation may be reflected in a change of chromatographic pattern. However, if a mutation resulted in a change in a quantitatively minor component in the cellular constituents, alterations in the chromatographic patterns would not be readily observed.

## SUMMARY

1. A procedure is described for the use of DEAE-cellulose chromatography in the separation and analysis of proteins in complex biological mixtures.

2. Examples are given of the chromatographic analysis of egg white, livetin fraction of egg yolk, chicken breast muscle extract, cabbage extract, and extracts of two different strains of *E. coli*.

3. It was found that, in the case of egg white and livetin fraction, patterns obtained by DEAE-cellulose chromatography show more detail than the patterns obtained by electrophoretic examination.

4. Some limitations of the procedure are discussed.

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# EIN ANALOGINTEGRATOR ZUR GASCHROMATOGRAPHISCHEN APPARATUR

Z. BÖHM

*Institut für Erdölforschung, Brno (Tschechoslowakei)*

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## EINLEITUNG

Die Entwicklung des Gaschromatographen ist neuzeitlich auf die Konstruktion der automatischen gaschromatographischen Apparatur gerichtet. Die Trennung der gasförmigen und flüchtigen Stoffe mittels Gaschromatographie einzelner Komponenten hinter der gaschromatographischen Kolonne wird mittels eines Detektors aufgenommen, der zum Beispiel auf dem Prinzip der Wärmeleitfähigkeit der Gase gegründet ist. Das Ausgangssignal des Detektors, das mittels eines Verstärkers verstärkt wird, wird zum Registriergerät geführt, welches einzelne Gaskomponenten durch Kurven vermerkt. Die Fläche dieser Kurven entspricht dem Inhalt der entsprechenden Gaskomponente. Zur Bewertung der Menge einzelner Gaskomponenten und deren gegenseitigen Verhältnisse ist die Auswertung der von der Kurve begrenzten Fläche vorzunehmen. Es wurden bereits einige Typen der Analogintegratoren auf verschiedenen Prinzipien[1-5] entwickelt. Ein Nachteil der bisher entwickelten Integratoren besteht darin, dass sie für den automatischen Betrieb nicht anwendbar sind.

## DAS PRINZIP DES GERÄTS

Den Grundteil des Integrators bildet ein Niederfrequenz-Tongenerator. Die Frequenz des NF-Generators wird mittels eines Reaktanzkreises betätigt und zwar auf die Weise, dass der Reaktanzkreis den Bestandteil des Oszillationskreises eines Oszillators des NF-Generators bildet. Wird zum ersten Gitter der Reaktanzröhre die Spannung zugeführt, so ändert sich die Reaktanz dieses Kreises und damit auch die Frequenz des Oszillators. Diese Abhängigkeit der Endfrequenz des NF-Generators von der Steuerspannung des ersten Gitters der Reaktanzröhre ist das Grundprinzip der Funktion des Integrators.

Wenn man in Betracht zieht, dass die durch den graphischen Vermerk des Verlaufes der Ausgangsspannung des Tastfühlers begrenzte Fläche ein Integral der Leitfähigkeit nach der Zeit ist, und wenn man sich vorstellt, dass die Frequenz der Ausgangsspannung aus dem NF-Generator im linearen Verhältnis zur Grösse dieser Spannung steht, dann ist die von der Anzahl der binnen gegebener Zeit $T = t_2 - t_1$ durchgegangenen Perioden linear abhängige Zahl auch linear abhängig vom Integral der Leitfähigkeit (Fig. 1). Die Addition der Periodenzahl wird im weiteren Teil der elektromagnetischen Nummerungseinrichtung, d.h. im Numerator vorgenommen[9–6].

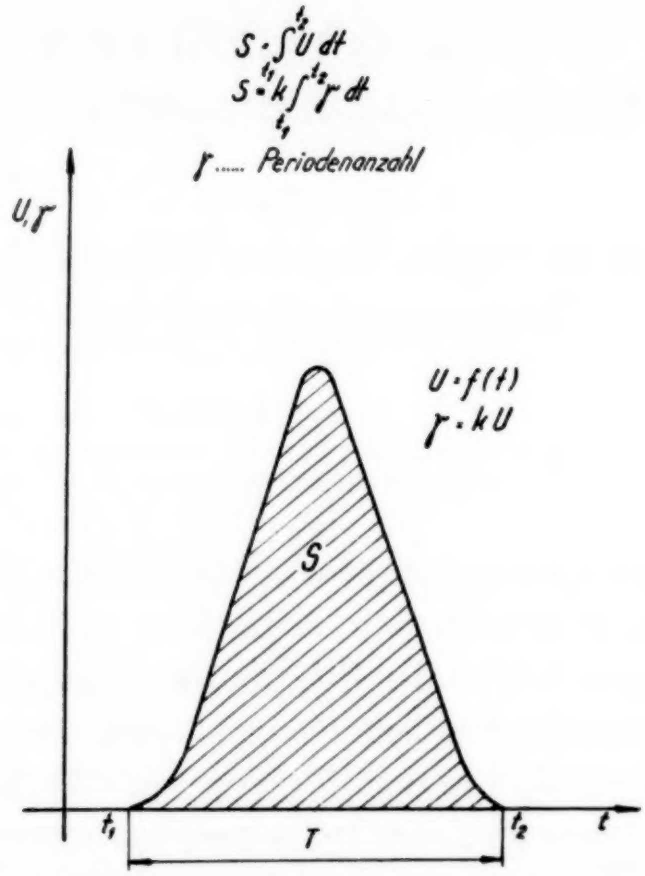
$S \cdot \int_{t_1}^{t_2} U\,dt$
$S \cdot k \int_{t_1}^{t_2} \Gamma\,dt$
$\Gamma$ ...... Periodenanzahl
$U, \Gamma$
$U \cdot f(t)$
$\Gamma \cdot kU$
S
$t_1$
T
$t_2$
t

Fig. 1. Vergleich des Verhältnisses der Anzahl der in gegebener Zeitspanne $T = t_2 - t_1$ durchgegangenen Perioden und des Verhältnisses des Wärmeleitfähigkeitsintegrals.

## DIE KONSTRUKTION DES GERÄTS

Der Analogintegrator besteht aus einem elektronischen Teil und einer elektromagnetischen Nummerungseinrichtung (Fig. 2).

### 1. *Die elektronische Einrichtung*

Der elektronische Teil enthält einen vom Widerstandskreis gesteuerten NF-Generator. Es wurde hier das Prinzip des Tongenerators verwendet. Es sind zwei selbständige Oszillatoren, die mit gleicher Frequenz von *ca.* 5 kc/sec arbeiten. An den Kondensator des Oszillationskreises des einen Oszillatoren ist ein zusätzlicher Kondensator der Reaktanz angeschlossen. Mit Hilfe dieses Blindwiderstandskreises kann die Frequenz des Oszillators im Bereich von 5000–5100 c/sec gewechselt werden. Beide Oszillatoren sind an einen Kreismodulator angeschlossen, an dessen Ausgang man die Differenz, sowie die Summe der Frequenzen beider angeschlossenen Oszillatoren erhält. Ändert man die Frequenz eines der Oszillatoren im Bereich von 5000 bis 5100 c/sec, so erhält man am Ausgang die Summe- und Differenzfrequenzzone. Die Summe-Zone wird mittels Filters abfiltriert (NF-Durchlassung), das aus Drosseln und Kondensatoren besteht. Am Ausgang des Filters erhält man also ein Sinussignal mit einer im Bereich von 0–100 c/sec lenkbaren Frequenz. Dieses Signal wird an zwei Frequenzverstärkungsstufen in Gegentaktschaltung zugeleitet. Die Anwendung der Gegentaktschaltung ist durch den symmetrischen Ausgang des Kreismodulators gegeben. Die zweite Ver-

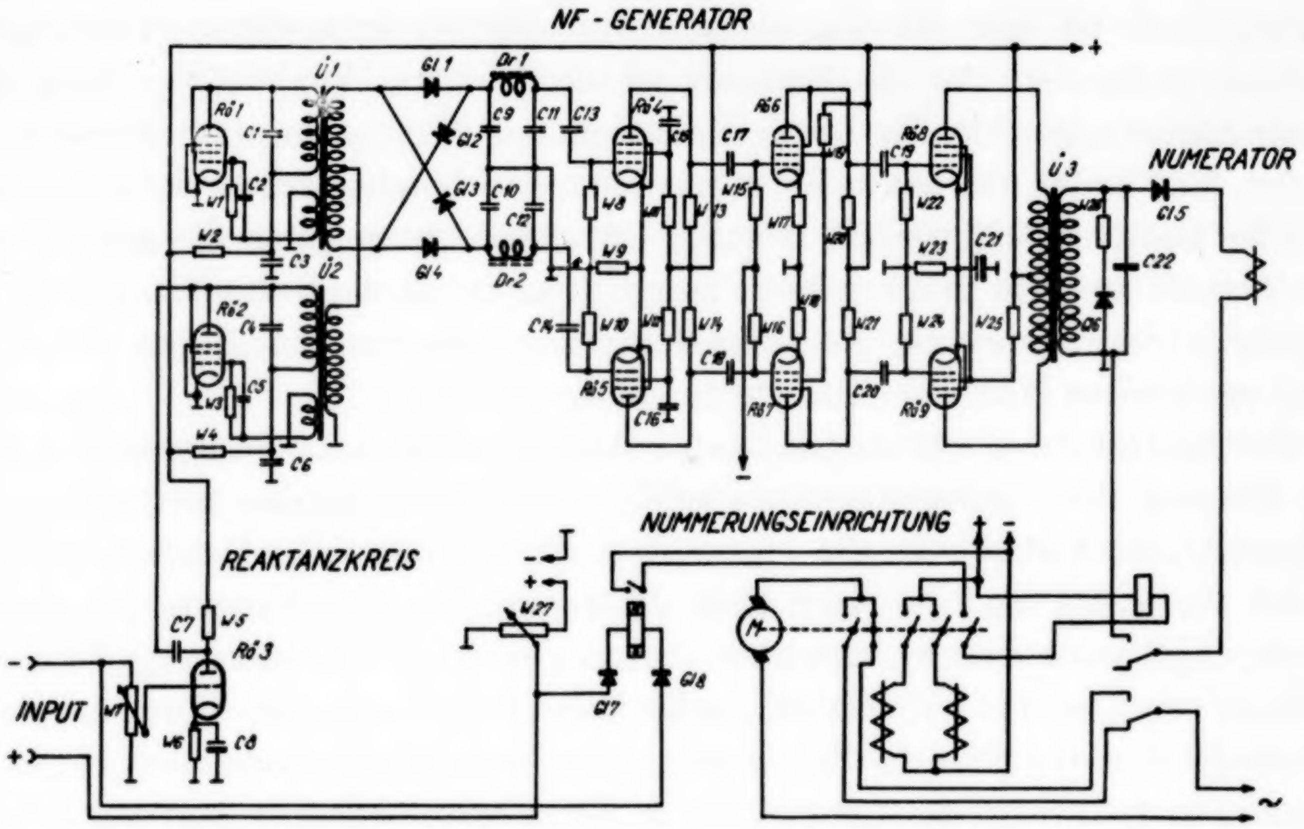
NF - GENERATOR
+
Ü1
Ü2
Ü3
Rö1
Rö2
Rö3
Rö4
Rö5
Rö6
Rö7
Rö8
Rö9
Gl1
Gl2
Gl3
Gl4
Gl5
Gl6
Gl7
Gl8
Dr1
Dr2
C1
C2
C3
C4
C5
C6
C7
C8
C9
C10
C11
C12
C13
C14
C15
C16
C17
C18
C19
C20
C21
C22
W1
W2
W3
W4
W5
W6
W7
W8
W9
W10
W13
W14
W15
W16
W17
W18
W21
W22
W23
W24
W25
W26
W27
NUMERATOR
NUMMERUNGSEINRICHTUNG
REAKTANZKREIS
INPUT
M
-
~

Fig. 2. Schema der Schaltung des Integrators.

| | | | | | |
|---|---|---|---|---|---|
| C1 | Kf.-Kond. | 0.02 μF 250 V | Rö1 | Röhre | EF12 |
| C2 | P.-Kond. | 0.01 μF 250 V | Rö2 | Röhre | EF12 |
| C3 | P.-Kond. | 1 μF 160 V | Rö3 | Röhre | EF36 |
| C4 | Kf.-Kond. | 0.02 μF 250 V | Rö4 | Röhre | EF12 |
| C5 | P.-Kond. | 0.01 μF 250 V | Rö5 | Röhre | EF12 |
| C6 | P.-Kond. | 1 μF 250 V | Rö6 | Röhre | EF14 |
| C7 | Kf.-Kond. | 0.01 μF 250 V | Rö7 | Röhre | EF14 |
| C8 | MP.-Kond | 25 μF 25 V | Rö8 | Röhre | EL41 |
| C9 | P.-Kond. | 0.05 μF 125 V | Rö9 | Röhre | EL41 |
| C10 | P.-Kond. | 0.05 μF 125 V | W1 | Sch.-Wd. | 100 kΩ |
| C11 | P.-Kond. | 0.05 μF 125 V | W2 | Sch.-Wd. | 5 kΩ |
| C12 | P.-Kond. | 0.05 μF 125 V | W3 | Sch.-Wd. | 100 kΩ |
| C13 | P.-Kond. | 0.1 μF 125 V | W4 | Sch.-Wd. | 5 kΩ |
| C14 | P.-Kond. | 0.1 μF 125 V | W5 | Sch.-Wd. | 6 kΩ |
| C15 | P.-Kond. | 0.5 μF 250 V | W6 | Sch.-Wd. | 320 Ω |
| C16 | P.-Kond. | 0.5 μF 250 V | W7 | Sch.-Dreh-Wd. | 100 kΩ |
| C17 | P.-Kond. | 0.5 μF 250 V | W8 | Sch.-Wd. | 2 MΩ |
| C18 | P.-Kond. | 0.5 μF 250 V | W9 | Sch.-Wd. | 500 Ω |
| C19 | P.-Kond. | 1 μF 250 V | W10 | Sch.-Wd. | 2 MΩ |
| C20 | P.-Kond. | 1 μF 250 V | W11 | Sch.-Wd. | 200 kΩ |
| C21 | MP.-Kond. | 25 μF 25 V | W12 | Sch.-Wd. | 200 kΩ |
| C22 | P.-Kond. | 0.05 μF 250 V | W13 | Sch.-Wd. | 50 kΩ |
| Gl1 | Germ. diode | 3NN40 | W14 | Sch.-Wd. | 50 kΩ |
| Gl2 | Germ. diode | 3NN40 | W15 | Sch.-Wd. | 500 kΩ |
| Gl3 | Germ. diode | 3NN40 | W16 | Sch.-Wd. | 500 kΩ |
| Gl4 | Germ. diode | 3NN40 | W17 | Sch.-Wd. | 100 Ω |
| Gl5 | Germ. diode | 3NN40 | W18 | Sch.-Wd. | 100 Ω |
| Gl6 | Germ. diode | 3NN40 | W19 | Sch.-Wd | 12.5 kΩ |
| Gl7 | Germ. diode | 3NN40 | W20 | Sch.-Wd. | 2.3 kΩ |
| Gl8 | Germ. diode | 3NN40 | W21 | Sch.-Wd. | 2.3 kΩ |
| Ü1 | Übertrager | | W22 | Sch.-Wd. | 1 MΩ |
| Ü2 | Übertrager | | W23 | Sch.-Wd. | 85 Ω |
| Ü3 | Übertrager | | W24 | Sch.-Wd. | 1 MΩ |
| Dr1 | Drossel | | W25 | Sch.-Wd. | 500 Ω |
| Dr2 | Drossel | | W26 | Sch.-Wd. | 50 Ω |
| M | Motor | 220 V 35 W | W27 | Sch.-Dreh.-Wd. | 3 kΩ |

stärkungsstufe ist mit der Leistungsendstufe mittels kapazitiver Widerstandskupplung verbunden. An die Endstufe ist die primäre Windung des Ausgangstransformators angeschlossen. Die Sekundärwindung des Ausgangstransformators ist mit dem Numerator über einen Einwegmodulator verbunden, sodass der Numerator bei jeder positiven Halbperiode arbeitet. Um die Belastung über die ganze Dauer einer Periode auf dem gleichen Wert zu erhalten, ist an die Sekundärwindung des Ausgangstransformators ein Belastungskreis angeschlossen, durch den dann der Strom der zweiten Hälfte der Periode durchfliesst.

*Der Reaktanzkreis.* Die mittels des Verstärkers verstärkte Steuerspannung wird zum Eingang des Analogintegrators geführt, und zwar an das Steuergitter der Reaktanzröhre. Ändert man die Spannung am Steuergitter der Reaktanzröhre im Bereich von 0 bis 2.5 V, so wird eben die notwendige Verstimmung des zweiten Schwingungskreises erreicht, damit am Ausgang des NF-Generators eine Frequenzänderung von 0 bis 100 c/sec erzielt werden kann. Der Eingang des Integrators kann für verschiedene Bereiche der Steuerspannung mittels Widerstandsteilers eingestellt werden. Der Eingangswiderstand ist 25 k$\Omega$. Bei maximaler Steuerspannung, d.h. bei 2.5 V, verursacht der Reaktanzkreis eine Verstimmung des zweiten Oszillators von 5 kc auf 5.1 kc/sec, sodass die Endfrequenz des NF-Generators 100 c/sec beträgt. Diese Abhängigkeit der Endfrequenz des NF-Generators von der Steuerspannung hat linearen Verlauf.

### 2. *Elektromagnetische Nummerungseinrichtung*

Die Steuerung der Nummerungseinrichtung erfolgt durch den Schaltkreis, der Anfang und Ende des Verlaufes der Umschlagskurve der integrierten Fläche angibt und die Funktion der Nummerungseinrichtung steuert. Den Hauptteil des Triggers bildet ein polarisiertes Mikrorelais. In einer Serie mit dem polarisierten Mikrorelais sind Germaniumdioden eingeschaltet. Durch die geeignete Einschaltung der Germaniumdioden wurde deren hoher Widerstand in undurchlässiger Richtung in Funktion eines Begrenzers des Stromes für das Mikrorelais ausgenutzt. Der Trigger ist parallel zum Reaktanzkreis an den Integratoreingang angeschlossen. Die Empfindlichkeit des Triggers ist 10 mV. Bei dieser Eingangsspannung koppelt das polarisierte Mikrorelais die Arbeitskontakte, die das Arbeitsrelais des Zählers einschalten. Es wird dann bei 9 mV am Integratoreingang ausgeschlatet. Der Strom für die verlässliche Verkopplung der Kontakte des polarisierten Mikrorelais ist 20 $\mu$A. Zwischen den Eingangsklemmen des Integrators und dem Reaktanzkreis mit dem parallel angeschlossenen Trigger ist ein Kreis zwischengeschaltet, mit Hilfe dessen mittels eines Regulationspotentiometers die Spannung der umgekehrten Polarität als die Steuerspannung am Integratoreingang eingestellt werden kann. Die Regelung des Potentiometers erfolgt vom Paneel der Apparatur und dient zur Einstellung der Nullspannung am Integratoreingang im Falle der Einstellung des Nullniveaus der Registrationsfeder am Registrator ausser grundsätzlicher Nullinie.

Sämtliche Arbeitsfunktionen der Nummerungseinrichtung (Druck, Papierschiebung und Nulleinstellung des Numerators) sind elektromagnetisch gesteuert.

Die Ein- und Ausschaltung einzelner Arbeitsfunktionen erfolgt mit Hilfe einer Daumennocke, die vom Elektromotor betrieben wird. Die Ein- und Ausschaltung des Elektromotors wird durch die Kontakte des Arbeitsrelais und des Daumenschalters gesteuert.

*Der Zähler der Nummerungseinrichtung.* Zur Konstruktion des Zählers wurde ein fünfstelliger Drehzähler angewendet, bei welchem die Zifferblatträder mit Gummistempelzahlen versehen wurden. Das Einheits-Zifferblattrad ist fest mit dem Schnapperzahnrad des elektromagnetischen Impulszählers verbunden. Bei jedem Impuls, d.h. bei jeder positiven Halbperiode des Sinusvorganges, die aus dem NF-Generator kommt, wendet sich ein wenig das mit Hilfe eines Sperrkranzes am Anker des elektromagnetischen Impulszählers befestigte Schnapperrad um einen Zahn um, was am Zähler einer Wendung des Einheits-Zifferblattrades um die Einheit entspricht. Der Drehzähler gibt also die Endsumme von Impulsen an, die der mit graphischem Vermerk begrenzten Fläche, die wir integrieren entspricht. Der Numerator is an die Sekundärwindung des Ausgangstransformators des NF-Generators über ein Paar Kontakte des Arbeitsrelais angeschlossen. Diese Kontakte sind nur während der Dauer der Integration einer Kurvenfläche eingeschaltet und verhindern also die Zusammenziehung der mit der entsprechenden Kurve nicht zusammenhängenden Impulse, z.B. bei Änderung der Polarität der Eingangs-Steuerspannung. Die mittlere Geschwindigkeit des Numerators ist 100 c/sec, d.h. 100 Einheiten an der Zählereinrichtung. Es ist möglich, durch geeignete Konstruktion des Numerators bis 200 Einheiten Zählgeschwindigkeit zu erreichen.

Der Druck ist am elektromagnetischen Prinzip so durchgeführt, dass der Anker des Elektromagnets die mechanische Einrichtung auslöst, welche den Papierstreifen (wie an Rechenmaschinen verwendet) über das Maschinenband an die Gummistempelzahlen des Numerators zudrückt. Der ziffermässige Endwert der Kurvenfläche wird auf den Papierstreifen abgedruckt. Gleichzeitig mit dem Druck erfolgt die Weiterschiebung des Maschinenbandes.

Die Schiebung des Papierstreifens und die Nullstellung des Numerators erfolgt ebenfalls elektromagnetisch auf die Weise, dass der Anker des Elektromagnets das Sperrkranzrad um einen gewissen Winkel mit Hilfe einer Hebeleinrichtung um etwas wendet. Das Sperrkranzrad is auf der Achse der Wickelhaspel für den Papierstreifen befestigt.

Die Nullstellung des Numerators erfolgt durch denselben Elektromagnet, welcher die Papierschiebung steuert und auch die mechanische Einrichtung für die Nullstellung des Numerators in Bewegung setzt. Die Schiebung und Nullstellung des Numerators erfolgen gleichzeitig.

Die Speisequellen für den Analogintegrator sind von üblicher Durchführung mit entsprechender Stabilisation der Anodenspannung für den NF-Generator.

## DIE FUNKTION DES ANALOGINTEGRATORS

Wie bereits erwähnt, wird die Nummerungseinrichtung mittels Triggers betätigt. Sobald die Steuerspannung am Integratoreingang 10 mV erreicht (verstärkte Indikationsspannung des Detektors), kuppelt das polarisierte Mikrorelais den elektrischen Stromkreis des Arbeitsrelais, das dann mit Hilfe weiterer Kontakte weitere Kreise der Nummerungseinrichtung einschaltet. Ein Paar Kontakte schliessen den elektrischen Kreis der Sekundärwindung des Ausgangstransformators des NF-Generators samt Numerator ab, welcher der Fläche unter der integrierten Kurve proportionale Impulse zusammenzurechnen anfängt. Nach Erreichung des Maximums der Steuerspannung sinkt die Steuerspannung wieder auf Null zurück. Bei Spannung von 9 mV schaltet das polarisierte Mikrorelais die Kontakte aus, wodurch der elektrische Stromkreis mit dem Arbeitsrelais unterbrochen wird. Die Kontakte des Arbeitsrelais werden abgestellt und der elektrische Kreis des Numerators mit dem NF-Generator wird somit unterbrochen. Weitere Kontakte des Arbeitsrelais setzen den Elektromotor in Bewegung, der den Daumenschalter betätigt. Der Daumenschalter schaltet mittels seiner Kontakte stufenweise einzelne Teile der Nummerungseinrichtung in nachstehender Folge ein und aus: 1. Er schaltet den Druck ein. 2. Er schiebt den Papierstreifen mit dem darauf gedruckten nummerischen Wert der Kurvenfläche und gleicht den Numerator auf den Nullstand ab. Weitere geeignete Einschaltung der Kontakte des Daumenschalters und des Arbeitsrelais im Laufe eines Nummerungszyklus bereitet die ganze Einrichtung für weitere Integration der nachfolgenden Kurvenfläche vor.

## ERGEBNISSE DER VERSUCHS- UND KONTROLLMESSSUNGEN

Das Ziel der Messungen war, den Prozentfehler des Analogintegrators festzustellen. Messungen wurden mit dem Industriegerät der Fa. Griffin & George, London, in folgender Schaltung vorgenommen: Quelle und Messung des Trägergases—die Referenz-Wärmeleitfähigkeitszelle—Probenprüfraum—chromatographische Kolonne—Zelle für Messungen der Wärmeleitfähigkeit. Die Wärmeleitfähigkeitszellen sind Durchflusszellen mit direktem Platinfaden, *ca.* 10 cm lang, geglüht mit 125 mA Strom aus einem 6 V-Akkumulator. Sie wurden an die Wheatstonebrücke angeschlossen, deren Ausbalancierung auf dem Honeywell-Brown Registrationsmillivoltmeter für 0–3 mV, mit Stufenleiter von 28 cm, angezeigt wurde; die Bewegung der Feder über die ganze Skala dauerte 2 Sekunden, die Geschwindigkeit der Papierschiebung war 1 cm/min. Chromatographische Kolonnen waren aus Glas aufgebaut, die Verbindung mit dem Detektor erfolgte mittels Schliffe mit Kapillaren. Mit Rücksicht auf die unterschiedliche Konstruktion und Speicherungsweise des Registriermillivoltmeters wurde ein Gleichstromverstärker verwendet[10,11]; mit Hilfe dieses Geräts konnte die Indikationsspannung der Leitfähigkeitszellen auf den Wert der Steuerspannung des Analogintegrators verstärkt werden. Als die zu analysierende Probe wurde das Heizgas verwendet, welches Propan, Isobutan, *n*-Butan und spurenweise auch Methan und Äthan enthielt. Als Trägergas wurde der Wasserstoff mit Druck von 750 mm Hg am Anfang der Kolonne und von 730 mm Hg am Ende der Kolonne bei 20° angewendet. Die Kolonne war mit Silikon + Dibutylphtalat mit 17 % Dibutyl auf Alusil gefüllt[12]. Die Dosierung der Probe erfolgte mittels Kolbenpipette (1 $cm^3$), die mit einer Injektionsnadel versehen war. Bei jeder Analyse erhielten wir vier Kurven, und zwar von rechts nach links: Luft, Propan, Isobutan, *n*-Butan. Der Anhaltspunkt zur Bestimmung des Messfehlers des Analogintegrators war die Voraussetzung, dass das Verhältnis der Kurvenfläche einzelner Fraktionen bei wiederholten Analysen konstant ist. Während einer Analyse wurden vier Kurven eingezeichnet und gleichzeitig wurde ihre Fläche mittels Analogintegrator ausgewertet. Zwecks Errechnung des Mittelfehlers einiger wiederholten Analysen wurde das Verhältnis der Kurvenfläche für das Isobutan zu derjenigen für das *n*-Butan, sowie das Verhältnis der Fläche für das Isobutan zu der für das Propan berechnet. Die Werte der Verhältnisse der Kurvenflächen wurden in die Tafel eingetragen und der mittlere Messfehler wurde berechnet. Um den abgemessenen Mittelfehler der Berechnung der Flächen mittels Analogintegrators zu vergleichen, wurde die Errechnung des Mittelfehlers nach der Maximalhöhe der Umschlagskurve durchgeführt. Diese Methode ist von den bisher angewendeten Berechnungsmethoden die genaueste. Nach Bewertung der abgemessenen Werte wurde der Mittelfehler für die Errechnung des Mittelfehlers der Verhältnisse nach der Maximalhöhe der Kurven berechnet, der 4.2 % betrug. Wenn der Wert des Mittelfehlers nach den Maximalhöhen mit dem durchschnittlichen Fehler der Berechnungen von Mittelfehler der Verhältnisse der Kurvenfläche verglichen wird, der 3.3 % gleich ist, so kann beurteilt werden, dass die Berechnung der Kurvenfläche mittels des Analogintegrators befriedigend ist, falls es in Betracht gezogen wird, dass sich im Endfehler von 3.3 % auch der Messfehler des Chromatographen, der 1 % beträgt, sowie der Fehler des Gleichstromverstärkers, der sich auch auf 1 % beläuft, inbegriffen sind.

## ZUSAMMENFASSUNG

Es wurde ein Gerät für die Messung der mit dem Diagramm des Verlaufes der Ausgangsspannung aus dem Indikator der gaschromatographischen Apparatur begrenzten Kurvenfläche entwickelt. Die Kurvenfläche bildet einen von den Parametern, der die Menge des im Gemisch flüchtiger Stoffe oder Gase enthaltenen Stoffes angibt. Es ist der Analogintegrator der empirischen Kurven gegebenen Typus, der direkte numerische Bewertung der Kurvenflächen vollzieht und ihren Wert mittels Drucks registriert. Dieses Gerät wurde als ein Bestandteil des automatischen Gaschromatographen konstruiert, vorläufig als selbständige Einheit der Apparatur. In der Konstruktion des automatischen Gaschromatographen bleibt noch eine Aufgabe für die Konstrukteure offen, und zwar die Registrationseinrichtung mit dem Analogintegrator in eine Apparatureinheit so zu vereinigen, dass der numerische Endwert der Kurvenfläche je bei der entsprechenden Kurve im Registrationsvermerk gedruckt werden kann.

## SUMMARY

An apparatus was designed for measuring the area below the curve recording the course of the output voltage of the indicator in gas chromatography. This area is one of the parameters indicating the amount of a substance present in a mixture of volatile substances or gases. The new device is an analogue integrator for empirical

curves of this type, which makes a direct numerical evaluation of the areas and records the values in print. The apparatus is designed to form a part of the automatic gas chromatograph, but at present it forms a separate unit. There is still a problem to be solved in designing gas chromatographs, that of combining the recorder with the analogue integrator in one unit in such a way that the values calculated for the various curve areas are printed at the appropriate curves.

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# Short Communications

## Ein Eluiergerät für die Papierchromatographie

Die wohl meist benutzte und genaueste Methode zur quantitativen Auswertung von Papierchromatogrammen beruht auf der Elution der getrennten Substanzen und Bestimmungen nach einem geeigneten physikalisch-chemischen Verfahren. Zwar ist dabei eine gewisse Absorbtion der Stoffe an die Papierfaser—je nach Natur der getrennten Substanzen—in Rechnung zu stellen, jedoch gelingt es in bestimmten Fällen diesen Fehler auf ein Minimum zu reduzieren.

Bei der Bearbeitung einer neuen Mikromethode zur quantitativen Bestimmung der Lupinus-Alkaloide[1] war es erforderlich, ein Eluiergerät zu schaffen, mit dem unter gleichen Bedingungen aus mehreren Filtrierpapierstreifen möglichst schnell Eluate hergestellt werden können. Diesen Anforderungen entsprachen leider nicht die gewöhnlich angewandten Geräte[2-6]. Ihre Unzulänglichkeit beruht hauptsächlich auf den Schwierigkeiten, welche beim Einhalten gleicher Elutionsbedingungen für

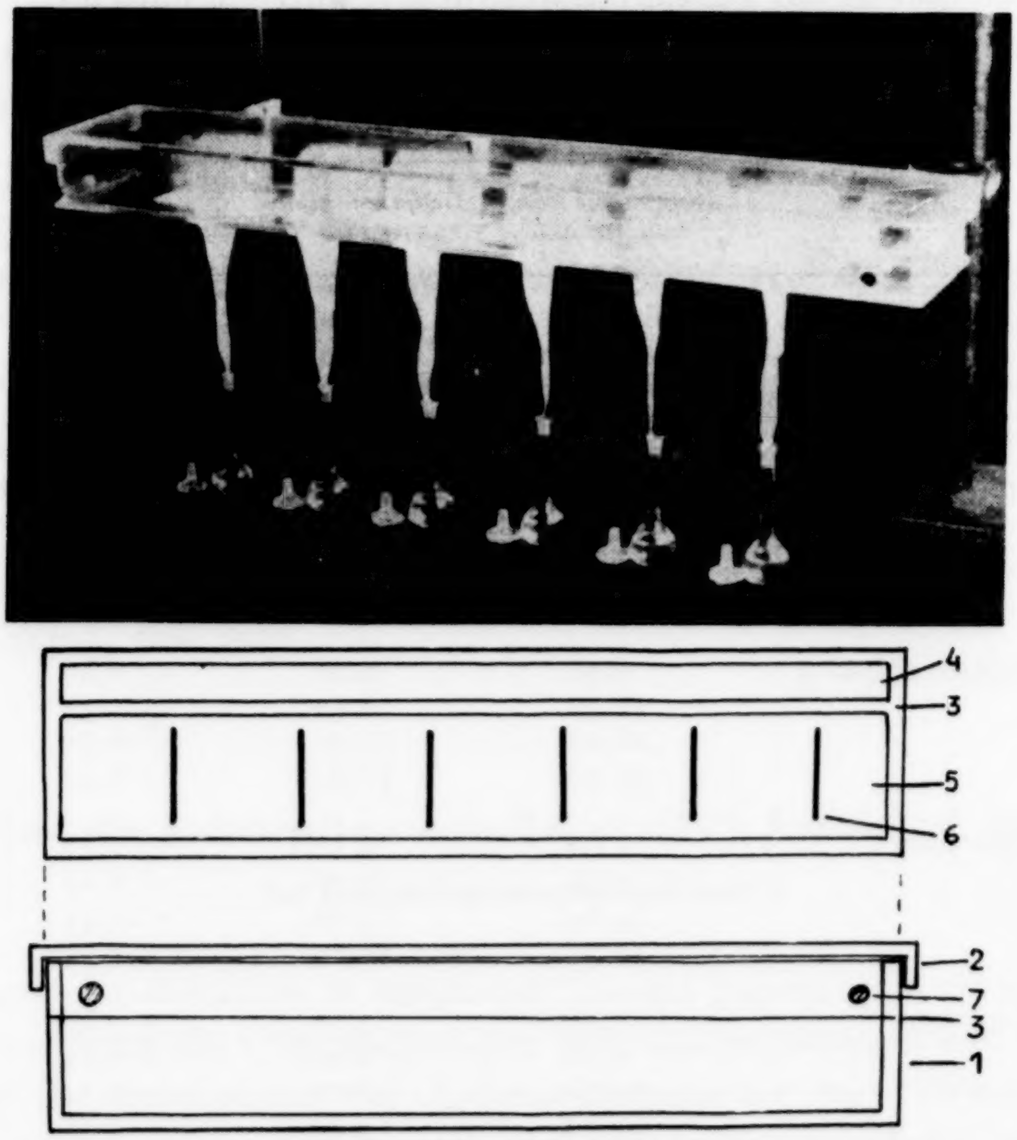

Fig. 1. Das Eluiergerät.

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eine grössere Anzahl von Substanzstreifen auftreten. Die unten beschriebene Apparatur, deren Ausmasse mühelos erweitert werden können, erlaubt es eine fast beliebige Anzahl Filtrierpapierstreifen zu eluieren. Die Elutionsdauer, welche hauptsächlich von der Natur der Stoffe und dem Lösungsmittel abhängig sein wird, lässt sich in grossem Massstab regulieren.

Das Gerät, welches aus 4 mm starken Plexiglas hergestellt wurde, besteht aus einer 500 × 100 × 80 mm grossen Kammer (1) mit passendem Deckel (2). Der Innenraum ist durch eine 50 mm hohe Platte (3) in zwei ungleich grosse Tröge getrennt. Der kleinere (4) hat eine innere Breite von 28 mm, der grössere (5) 60 mm. Im Boden des grösseren Trogs befinden sich in gleichmässigem Abstand eingestanzte 1 mm breite und 40 mm lange Schlitze (6) zur Aufnahme der Papierstreifen. Die Seitenwände der Kammer enthalten zwei Bohrungen vom ø 10 mm (7) durch die passende Glas- oder Metallstäbe geführt werden, welche es ermöglichen das Gerät auf zwei gewöhnlichen Stativen in beliebiger Höhe aufzuhängen.

Die Bedienung des Elutionsgerätes ist sehr einfach. In die einzelnen Schlitze führt man je zwei Filtrierpapierstreifen, deren obere Enden von einander abgebogen werden. Die unteren Enden der Streifen sind soweit zugespitzt, dass ihre Breite den aus den Chromatogrammen ausgeschnittenen Streifen entspricht. Es kamen dicke Filtrierpapiersorten von grosser und mittlerer Saugfähigkeit, wie Schleicher & Schüll 2315 und Whatman No. 3 zur Anwendung. Der schmälere Trog wird nun mit Lösungsmittel beschickt und durch Filtrierpapierbrücken mit den einzelnen Streifenpaaren

## TABELLE I

### ALKALOIDWERTE

| *Versuch No.* | *Alkaloid* | *Aufgetragene Menge µg* | *Gefundene Menge µg* | *Differenz* | |
|---|---|---|---|---|---|
| | | | | *µg* | *%* |
| 1 | Lupanin | 4.96 | 4.90 | — 0.04 | — 0.80 |
| 2 | | 9.92 | 9.60 | — 0.22 | — 2.22 |
| 3 | | 14.88 | 14.50 | + 0.31 | + 2.08 |
| 4 | | 19.84 | 19.50 | — 0.34 | — 2.71 |
| 5 | Hydroxylupanin | 10.56 | 10.20 | — 0.36 | — 3.40 |
| 6 | | 21.16 | 20.90 | — 0.26 | — 1.23 |
| 7 | | 31.68 | 31.20 | — 0.48 | — 1.51 |
| 8 | | 42.24 | 42.00 | — 0.24 | — 0.57 |
| 9 | Angustifolin | 9.36 | 9.10 | — 0.26 | — 2.77 |
| 10 | | 18.72 | 18.40 | — 0.32 | — 1.71 |
| 11 | | 28.08 | 27.50 | — 0.51 | — 1.81 |
| 12 | | 37.44 | 37.20 | — 0.24 | — 0.64 |
| 13 | Spartein | 4.68 | 4.50 | — 0.18 | — 3.85 |
| 14 | | 9.36 | 9.10 | — 0.26 | — 2.77 |
| 15 | | 14.04 | 13.60 | — 0.44 | — 3.13 |
| 16 | | 18.72 | 18.20 | — 0.52 | — 2.77 |
| 17 | Lupinin | 3.38 | 3.10 | — 0.08 | — 2.36 |
| 18 | | 6.76 | 6.90 | + 0.14 | + 2.07 |
| 19 | | 10.14 | 10.00 | — 0.14 | — 1.38 |
| 20 | | 13.52 | 13.20 | — 0.32 | — 2.36 |

verbunden. Durch Änderung der Breite und Filtrierpapiersorte dieser Brücken kann die Elutionsdauer reguliert werden. Nach Aufsetzen des Deckels und Abtropfen einer kleinen Menge des Lösungsmittels, ist die Apparatur zur Aufnahme der Substanzstreifen bereit. Diese werden an ihrem unteren Ende zugespitzt und an die herunterhängenden feuchten Streifenpaare gedrückt. Das Eluat kann in entsprechenden Gefässen—meistens in Messkölbchen—gesammelt werden. Bei Anwendung flüchtiger organischer Lösungsmittel muss die gesamte Vorrichtung mit einer Glasglocke zugedeckt, oder in eine Chromatographiekammer gestellt werden. Die genaue Elutionsdauer muss jeweils durch Vorversuche ermittelt und eingestellt werden.

Die Apparatur wurde bei einer Mikromethode zur Bestimmung der Lupinus-Alkaloide angewandt und erprobt. Die Bestimmung wurde mit Hilfe der Bromthymolblau-Reaktion kolorimetrisch ausgeführt, welche mit einem Fehler von ± 3.0% belastet war[1]. Wässrige Standartlösungen von Alkaloidhydrochloriden wurden auf 15 × 80 mm grosse Schleicher & Schüll 2315-Filtrierpapierstreifen aufgetragen, die Substanz eluiert und in den einzelnen Eluaten die Alkaloidbestimmung vorgenommen. Als Auswaschflüssigkeit kam Wasser zur Anwendung, wobei die Laufzeit durch 40 mm breite Schleicher & Schüll 2315-Filtrierpapierbrücken auf 30 min festgelegt wurde. Das Eluatvolumen betrug in diesem Falle jeweils ungef. 4 ml und die Elution konnte—auch bei den grössten aufgetragenen Alkaloidmengen—als vollkommen betrachtet werden.

Aus der angeführten Tabelle ist ersichtlich, dass bei der Elution nur geringe Stoffverluste zu verzeichnen sind, welche die Fehlergrenzen der angewandten quantitativen Bestimmungsmethode nicht überschreiten.

*Institut für Allgemeine Chemie W.S.E. und Biochemische Abteilung des Instituts für Pflanzenzüchtung der Polnischen Akademie der Wissenschaften, Poznań (Polen)*

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# Über die Adsorption von Tellur (IV), Jodid und Jodat an Anionenaustauschern

Das Verhalten vieler Elemente in salzsaurer Lösung gegenüber Anionenaustauschern ist insbesondere durch die Untersuchungen von KRAUS, NELSON und Mitarbeitern[1,2] bekannt. Für die Anwendung der Anionenaustauscher zur Trennung von Spaltprodukten fehlen allerdings Angaben über das Verhalten von Tellur und Jod, zweier

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wichtiger Komponenten des Spaltproduktgemischs. Beim Jod interessiert zudem das Verhalten der verschiedenen Wertigkeitsstufen. Kraus und Nelson[1] bezeichnen Tellur(IV) als "stark adsorbierbar", für Jodid werden vorläufige Verteilungskoeffizienten mitgeteilt, während für Jodat keine Angaben gemacht werden. Blasius und Wachtel[3] haben beobachtet, dass elementares Jod von Anionenaustauschern sehr fest gebunden wird und nur nach Reduktion mit $SO_2$-haltiger HCl eluiert werden kann. Gregor, Belle und Marcus[4] haben die Adsorption von $J^-$ und $JO_3^-$ unter anderen Gesichtspunkten und Bedingungen studiert.

Wir haben die Adsorption von Tellur(IV), Jodid und Jodat am stark basischen Anionenaustauscher Dowex-1 X 10 untersucht. Dabei wurden radioaktive Indikatoren verwendet. Im Kernreaktor bestrahltes elementares Tellur wurde in HCl–$HNO_3$ gelöst und das aus $^{130}$Te$(n,\gamma\beta^-)$ entstandene $^{131}$J nach Zusatz von $J^-/JO_3^-$-Träger als $J_2$ abdestilliert. Der Rückstand—ein Gemisch mehrerer Tellurisotope—wurde für die Tellurversuche benutzt. Ein Teil des Destillats wurde mit $SO_2$ reduziert und für die Jodidversuche verwendet. Zu einem anderen Teil des Destillats wurden äquivalente Mengen $J^-/JO_3^-$ gegeben, angesäuert und das freiwerdende $J_2$ durch $Cl_2$-Gas und $KClO_3$ über JCl zu $JO_3^-$ oxydiert[5].

Mit diesen Lösungen wurden die Verteilungskoeffizienten $K_D$ (Aktivität pro g trockener Austauscher/Aktivität pro ml Lösung) in Abhängigkeit von der Salzsäurekonzentration bestimmt und nach Kraus und Nelson[2] die Volumenverteilungskoeffizienten $D_v$ (Aktivität pro ml Harzbett/Aktivität pro ml Lösung) berechnet. Auf eine zur Gleichgewichtseinstellung ausreichende Schütteldauer wurde geachtet. Die Gehalte der Lösungen an Träger waren im Vergleich zur Harzkapazität vernachlässigbar klein. Die Aktivitätsbestimmungen erfolgten durch $\gamma$-Zählung mit einem NaJ-Bohrlochkristall.

Fig. 1 zeigt log $D_v$ in Abhängigkeit von der Salzsäurekonzentration. Für $J^-$ ist diese Abhängigkeit qualitativ gleich der von Krauss und Nelson[1] beobachteten, unsere $D_v$-Werte sind jedoch um den Faktor 2–3 grösser. Die $D_v$-Werte für $J^-$ und $JO_3^-$ in konzentrierter HCl sind fast gleich gross; man darf wohl annehmen, dass eine teilweise Reduktion des $JO_3^-$ zu $J^-$ stattgefunden hat.

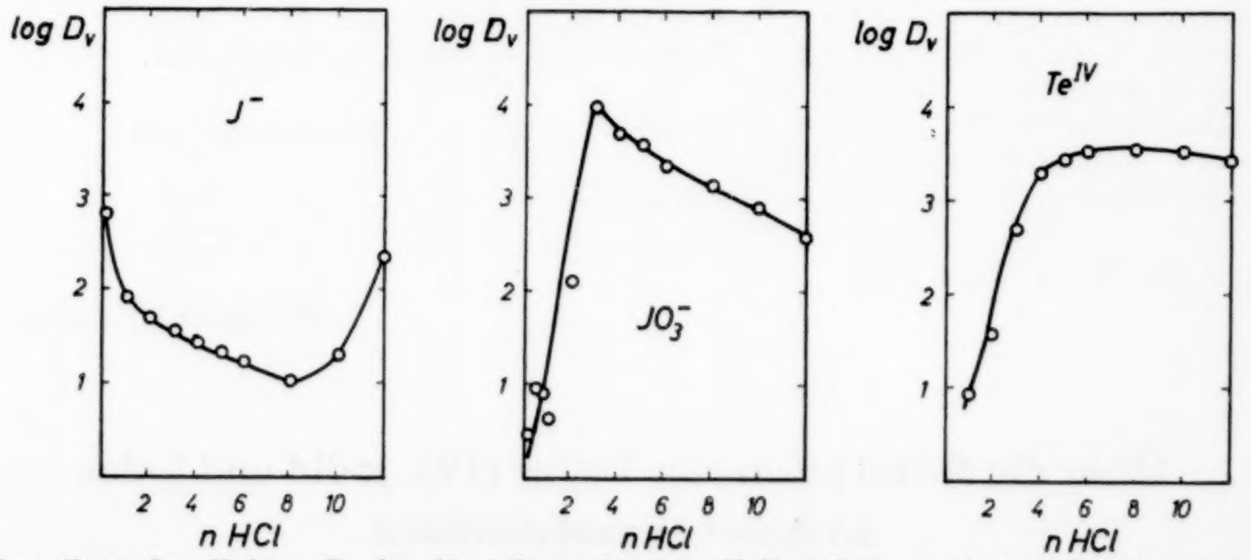

Fig. 1. Verteilungskoeffizient $D_v$ für die Adsorption von Tellur (IV), Jodid und Jodat am Anionenaustauscher Dowex-1 X 10, aufgetragen in Abhängigkeit von der Salzsäurekonzentration.

Es ist bekannt, dass das Verhalten mancher Elemente an Anionenaustauschersäulen nicht dem nach den $D_v$-Werten erwarteten entspricht (vgl. z.B.[2]). Bei den hier behandelten Elementen haben wir folgende Erfahrungen gemacht. Aus starker HCl adsorbiertes Tellur(IV) lässt sich mit wenigen Säulenvolumina 1 *N* HCl glatt eluieren. Für die Trennung des Tellurs von manchen Elementen ist es wertvoll, dass das Tellur auf der Säule durch 4–6 *N* HCl–$SO_2$ zum Element reduziert werden kann. Nach der Elution anderer Elemente mit HCl niedriger Konzentration kann das Tellur mit 8 *N* HCl–$HNO_3$ wiederum oxydiert und mit 1 *N* HCl eluiert werden.

Bei Säulenversuchen mit Jod treten häufig Schwierigkeiten auf. Ein aus starker, $Cl_2$-haltiger HCl adsorbiertes $JO_3^-$ kann zwar, wie erwartet wird, mit 0.01 *N* HCl eluiert werden, die Ausbeute beträgt jedoch meist nur etwa 50% und schwankt zudem stark. Die Elution als $J^-$ durch 6–8 *N* HCl–$SO_2$ ist auch nach Aufgabe als $JO_3^-$ möglich, sie erfolgt aber in der Kälte recht schleppend. Die Anwendung höherer Temperaturen oder niedrig vernetzter Harze ist vorteilhaft. Wegen des ungünstigen Verteilungskoeffizienten für $J^-$ sind auch dann noch relativ grosse Volumina HCl–$SO_2$ erforderlich. Wesentliche Unterschiede im Verhalten des Jods in oxydierten wie reduzierten Lösungen bei Gegenwart oder Abwesenheit von Träger haben wir nicht beobachtet.

Herrn Prof. Dr. F. STRASSMANN danken wir für sein freundliches Interesse, dem Bundesministerium für Atomkernenergie und Wasserwirtschaft für finanzielle Unterstützung.

*Zusatz bei der Korrektur*

Inzwischen wurde uns bekannt, dass U. SCHINDEWOLF (*Ann. Progr. Rept. Mass. Inst. Technol.*, 1955/56 und pers. Mitt.) die Adsorption von Te(IV) und Te(VI) an Anionenaustauschern untersucht hat. Die von SCHINDEWOLF für Te(IV) gefundene Abhängigkeit des Verteilungskoeffizienten von der HCl-Konzentration entspricht qualitativ der von uns beobachteten. Quantitativ stimmen die Werte nur bis etwa 5*N* HCl überein, bei höheren Konzentrationen fand SCHINDEWOLF etwa um den Faktor 5 grössere Verteilungskoeffizienten.

*Anorganisch-chemisches Institut, Universität Mainz*
(*Deutschland*)

EMIL KLEEMANN
GÜNTER HERRMANN

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# The preservation of paper chromatograms sprayed with ninhydrin

Excellent colour differentiation on chromatograms of amino acids is obtained by spraying with a solution of ninhydrin in chloroform containing a small amount of collidine[1] using mild heating conditions for development (about 2–3 min at 80°). Under these conditions, colours are seen which differ in many instances both in hue and intensity from the purple colours given by the majority of amino acids in the absence of collidine. These characteristic colours, which are often of use in confirming the presence of known substances or in revealing lack of homogeneity of incompletely resolved spots, usually fade to a uniform purple colour within 5–10 min under normal laboratory conditions. For this reason comparisons between large numbers of chromatograms are difficult and the comparing of chromatograms prepared and sprayed at different times is also precluded. It is possible to retard considerably the colour changes by sealing the chromatogram in a polythene bag immediately it has been developed. This effectively reduces loss of residual collidine, the presence of which appears to be essential both for the differential colouring and the stability of the colours. At the same time the chromatogram is protected from the atmosphere of the laboratory, which often hastens colour destruction. It has not been found satisfactory to develop the chromatogram in the bag, nor is there any advantage in increasing the collidine content of the spray beyond 0.2 %. The stability of the colours, though much improved by this technique is still affected by light and raised temperature. However, if the chromatograms are stored in the dark at about 4° it is possible to retain the original colours for up to 14 days. After this period the spots gradually assume the more usual purple colours, but here again, continued storage in the cold and away from light leads to a considerable increase in stability. Chromatograms have been successfully stored in this way for up to 3 months with little change in the intensity or colour of the spots. The spray we have found most successful is prepared by adding ninhydrin (200 mg) to collidine (0.2 ml), in which it dissolves completely, and making to 100 ml with chloroform. The method of development is critical, the chromatogram being removed from the heating oven immediately visual inspection indicates colour differentiation is optimal. At temperatures much above 80° this stage is reached so rapidly that it is difficult to obtain satisfactory results. The chromatograms are then placed immediately in the polythene bags which are closed by turning over the tops and sealing with adhesive tape.

*Wellcome Research Laboratories, Biological Division,*
*Beckenham, Kent (Great Britain)*
A. J. Woiwod

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# Anion exchange studies

## XXIX. Adsorption of $GeCl_4$ from gas streams*

Germanium may be adsorbed from concentrated hydrochloric acid solutions by strong base quaternary amine anion exchange resins[4–6]. Unfortunately, under the conditions where Ge(IV) is effectively adsorbed, it is very volatile and hence there is considerable danger of losing significant amounts in analytical procedures involving anion exchange in HCl solutions. As described recently[2], this difficulty may be circumvented by operating in HCl–HF mixtures in which the volatility of Ge(IV) is low while conditions for good adsorption exist.

The high volatility of Ge(IV) in concentrated HCl solutions has often been used for analytical purposes[7]. The procedures normally involve distillation followed by condensation to recover Ge(IV). A high temperature gas-sweeping technique was used more recently to separate a short-lived isomer of Ge ($^{73m}$Ge, $T_{\frac{1}{2}} = 0.52$ sec) from its long-lived $^{73}$As parent[8].

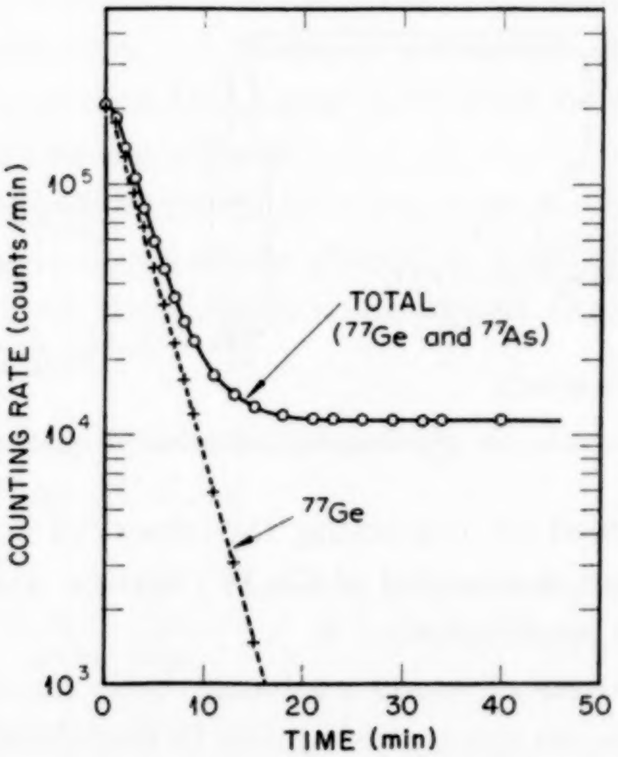

Fig. 1. Separation of $^{77}$Ge from $^{77}$As by gas-sweeping (12 *M* HCl, 25°).

It appeared that the analytical technique could be simplified (1) by using low temperature gas-sweeping techniques for removal of Ge(IV) from HCl solutions, and (2) by adsorbing Ge(IV) from the gas stream on an anion exchanger.

Low temperature gas sweeping permits essentially quantitative removal of Ge(IV) from concentrated HCl solutions as illustrated in Fig. 1. A 3 ml sample of

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* This document is based on work performed for the U. S. Atomic Energy Commission at the Oak Ridge National Laboratory, Oak Ridge, Tennessee, operated by the Union Carbide Corporation. Previous papers: XXVI, XXVII and XXVIII, see refs. 1–3.

12 *M* HCl containing $^{77}$Ge tracer*, in secular equilibrium with its daughter $^{77}$As, was placed in a small test tube fitted for gas sweeping with an inlet tube extending to the bottom of the test tube and with an outlet tube. The tube was placed in a well-type scintillation counter so that its counting rate could be followed continuously as nitrogen gas, previously washed with 12 *M* HCl, was passed through it.

The counting rate (corrected for "room" background) of the sample as a function of time is shown as a semi-log plot in Fig. 1. In this experiment a constant residual counting rate was reached in approximately 20 min. The remaining activity was found by radiochemical analysis to be $^{77}$As. The rate of removal of $^{77}$Ge, shown by the dashed line in Fig. 1, was obtained by correcting the observed counting rate for the $^{77}$As background. The half-time of removal of Ge(IV) was *ca.* 2 min. which is sufficiently short for most analytical applications.

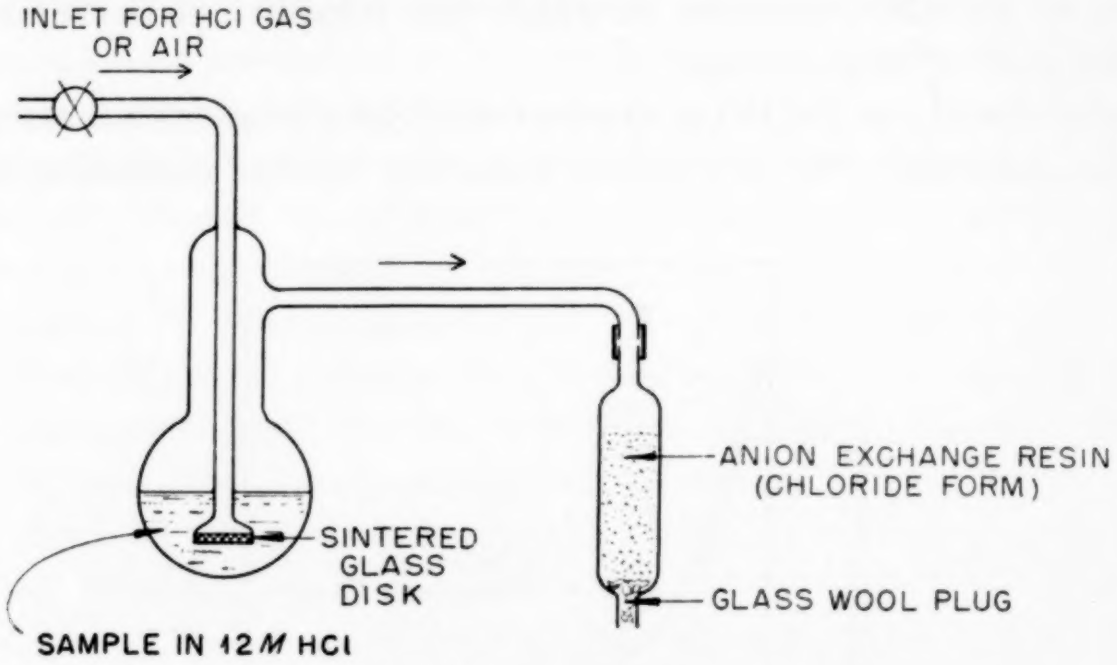

Fig. 2. Apparatus for distillation and adsorption of germanium.

To test the effectiveness of anion exchange beds for removing Ge(IV) ($GeCl_4$) from HCl-rich gas streams, an apparatus similar to that illustrated in Fig. 2 was used. The sample containing $^{77}$Ge in 12 *M* HCl was placed in a 5 ml distilling flask, connected by a side arm to two 0.25 cm$^2$ × 4 cm beds of resin (Dowex-1 X 8, 50–100 mesh, capacity 3.4 moles per kg dry resin) arranged in series. The resin columns had previously been washed with distilled water and centrifuged for several minutes to remove most of the interstitial water. Nitrogen gas, washed with a large excess of 12 *M* HCl, was bubbled through the system at a moderate rate. After allowing sufficient time for quantitative removal of Ge(IV) from the solution (*ca.* 20 min) the columns were counted in a well-type counter. There was essentially quantitative adsorption of all Ge(IV) in the first column; no Ge-activity was detected in the second column.

Hydrochloric acid apparently is also strongly adsorbed from the gas stream by the anion exchange bed. This may readily be demonstrated by holding moist litmus paper in the exit gas stream or, more simply, by following a characteristic color change (yellow to amber) which this batch of resin shows on being saturated with HCl. Large amounts of HCl may be adsorbed by the ion exchanger and the frontal edge is sharp even with relatively rapid gas flow. With water-washed resins, the amount of HCl held was approximately 0.2 g per c.c. of bed corresponding to *ca.* 6 moles HCl per liter of bed.

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* The tracer, $^{77}$Ge–$^{77}$As ($T_{\frac{1}{2}}$ = 12 h and 40 h), was prepared by irradiating $GeO_2$ in the ORNL Low Intensity Test Reactor (LITR). The irradiated oxide was dissolved in 0.5 *M* NaOH to prepare a "stock" solution.

If gas sweeping is continued until the resin column is saturated with HCl, there is danger of substantial loss of Ge(IV). For example, in one experiment gas sweeping was continued until the band of adsorbed HCl had reached the middle of the second column. Under these conditions *ca.* 20% of the $^{77}$Ge activity was transferred to the second column and only 80% was retained by the first one.

One of the many advantages of the gas sweeping-anion exchange technique is the ease with which Ge(IV) may be recovered from the anion exchange columns. Desorption may readily be achieved by washing the columns with a few column volumes of water or, preferably, of dilute HCl solution. Since the first part of the effluent is rather concentrated in HCl, some care must be exercised to avoid loss of Ge(IV) by volatilization from the effluent.

The small scale technique described for separation and isolation of Ge(IV) should be adaptable to large scale applications provided precautions are taken to avoid complications resulting from the limited solubility of Ge(IV) in concentrated HCl solutions[6] or in resin columns[5].

*Chemistry Division, Oak Ridge National Laboratory,*
*Oak Ridge, Tenn. (U.S.A.)*

Frederick Nelson
Kurt A. Kraus

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## Plastic bag technique for paper chromatography

Conventional paper chromatography generally employs microgram quantities of the compounds to be separated per sheet of paper. Column chromatography is used for larger amounts. However, cellulose powder columns produce broader and not as well defined zones as separations on filter paper, which is more uniform in structure.

In an attempt to separate larger amounts of material with the definition given by filter paper, Mitchell and Haskins[1] developed the "chromatopile" method. The detection of the zones required the removal of many sheets from the pile. Porter[2] modified this technique and clamped stacks of accurately cut paper strips to form a "chromatopack". The "Isolierpack" of Fischer and Behrens[3] was a further modification. The sample was applied to each of the strips of paper before they were accurately lined up for elution, a time-consuming procedure. Zechmeister[4] used a glass column packed with filter paper discs. Unless a precision bore tube and precision-punched papers were used "channeling and gross irregularities in the flow of solutions became manifest". Danielson[5] and Hagdahl and Danielson[6] described paper columns for preparative work, using filter paper tightly wound around an inert cylindrical rod and pressed into an outer cylinder of polythene. Special machines would be required to obtain close packing, which is a prerequisite for sharp separations. Brownell, Hamilton and Casselman[7] introduced the heavy paper technique. The sample is applied to Whatman seed-test paper as a uniform streak but in quantitative work difficulties were experienced in applying a predetermined volume uniformly.

To overcome these drawbacks, the author attaches a plastic bag to the seed-test paper (Fig. 1) through which sample and eluent are applied by gravity as in column chromatography . Fractions may be collected from a drip point or left as zones on the paper. This technique combines the simplicity of sample addition as in column chromatography with the advantages of uniformity of the filter paper.

For ascending elution, after the sample has been applied and washed into the heavy paper, the strip is turned upside down as shown in Fig. 2. Another possibility

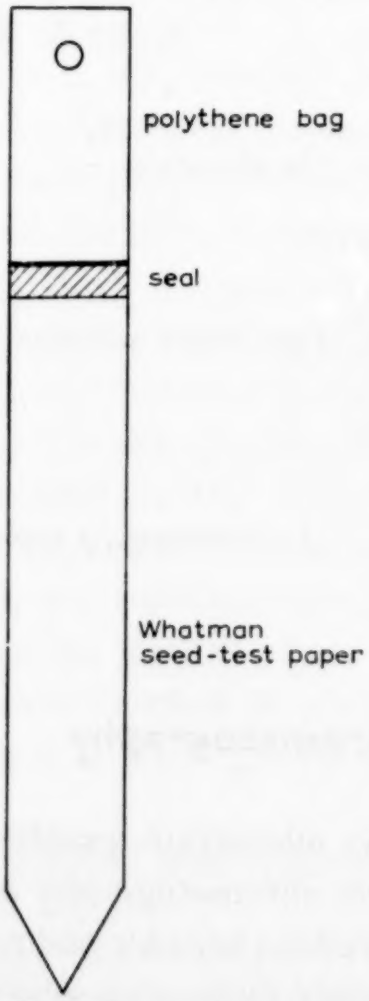

Fig. 1a

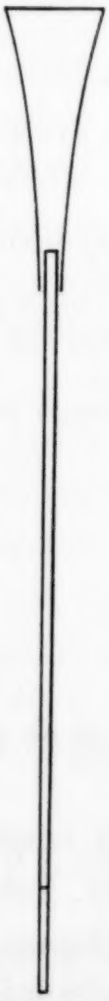

Fig. 1b

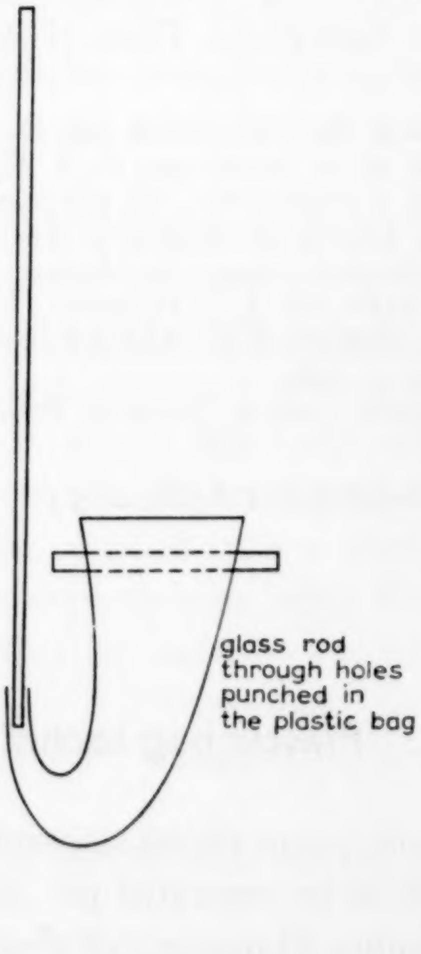

Fig. 2

would be, after the sample has been applied and washed in, to cut the plastic bag off and to proceed in the usual manner.

"Lay-flat" polythene tubing 2–3 inches wide and 0.005 inches thick proved most suitable for our purposes. However, other sizes may also be used.

Whatman seed-test paper is cut to suit the tubing available and to provide a tight fit when slipped about one quarter to half an inch into the tubing. The polythene is heat-sealed onto the paper, using a conventional strip sealing machine. A soldering iron run at reduced voltage produces an equally good seal, provided silicone grease is used to prevent the polythene from sticking to the copper tip.

Alternative but less satisfactory means of attaching the polythene tubing to the paper are clamping, sewing and sealing the stitches, or using an adhesive for polythene, which must not penetrate into the paper sufficiently to cause a blockage. Furthermore, the adhesive must not be soluble in the solvents subsequently used.

Fig. 3 shows the separation of a mixture of dyes by this technique. 5 ml of a water solution containing 5 mg each of Naphthalene Orange, Solway Blue, Azo Geranine, Fluorescein, Lissamine and Coomassie were simply pipetted into the polythene bag. Elution was done with water.

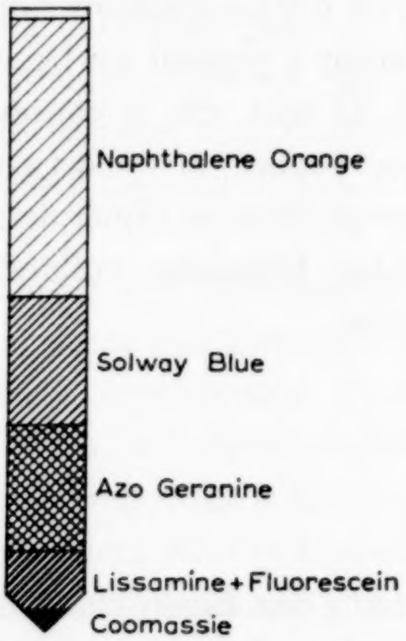

Fig. 3.

The author thanks Imperial Chemical Industries of Australia and New Zealand Limited for permission to publish this communication.

*Central Research Laboratories, ICIANZ Limited,* D. GUNEW
*Ascot Vale, Victoria (Australia)*

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## BOOK REVIEWS

*Anorganische qualitative Mikroanalyse* (Monographien aus dem Gebiete der qualitativen Mikroanalyse, herausgegeben von A. A. BENEDETTI-PICHLER, Band 1), par H. MALISSA ET A. A. BENEDETTI-PICHLER, Springer Verlag, Vienne, 1959, vii + 333 pages, 55 figures, relié toile, prix $ 11.65, DM 49.—.

On nous annonce une nouvelle collection de manuels relatifs à la microanalyse et à la recherche des traces; le présent ouvrage est le premier de la collection et semble convenir aussi bien à l'étudiant qu'au chercheur. Il traite seulement des ions et de quelques molécules de chimie minérale et se divise en trois parties: 1. Méthodes générales de travail (prélèvement, traitement, centrifugation, filtration, électrolyse, extraction, sublimation, vaporisation, distillation); 2. Les méthodes particulières de recherche des ions (en général, celles qui sont sélectionnées dans le 2ème Rapport de l'Union internationale et avec moins de détails); 3. Séparations des cations et des anions; chaque paragraphe est complété par une bibliographie choisie.

Sans présenter une grande originalité sur les livres existant actuellement dans le même sujet celui-ci est intéressant car il rassemble des éléments dispersés et les auteurs les présentent bien. Si l'on relève quelques fautes dans les noms propres, on doit remarquer que Springer Verlag a produit un beau livre, presque luxueux où les photographies sont bien venues. Il faut voir le contenu des tomes suivants pour se rendre compte des services que rendra la collection aux microanalystes; il faut convenir que pour cette partie qualitative minérale nous étions déjà bien pourvus tout au moins en langue anglaise, française, hollandaise et espagnole. Souhaitons donc un bon départ à la collection.

CLÉMENT DUVAL (Paris)

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*Seltene Naturstoffe, 1959/60*, Anhang mit Eigenschaften, Synonyma, Literaturangaben und papierchromatographischen Spezialmethoden. Published by Fluka A.G., Buchs S.G., Switzerland and Carl Roth, Karlsruhe, Germany, 83 pages.

This catalogue of rare natural organic compounds contains several short chapters on chromatographic techniques. Although provided with excellent illustrations and presented both in German and English, it gives little that has not already been dealt with in the literature. The apparatus recommended is unsuitable for two-phase solvents since no provision is made for placing the second phase in the container. For preparative purposes paper-disc chromatograms are proposed essentially as in previous publications on this topic. The only interesting feature is the list of rare compounds with physical properties (including $R_F$ values) for each substance.

# ÜBER DAS CHROMATOGRAPHISCHE VERHALTEN VON HYDROXYLHALTIGEN CARBONSÄUREN UND PHOSPHORSÄUREESTERN AN ALUMINIUMOXYD

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HERBERT GRASSHOF
*Chemisches Laboratorium der Firma M. Woelm, Eschwege (Deutschland)*

(Eingegangen den 11. Juni 1959)

Die starke Adsorptionsfähigkeit von Aluminiumoxyd, die durch van der Waals'sche Kräfte bedingt, in organischen Lösungsmitteln für die chromatographische Analyse ausgenutzt wird, zeigt sich auch bei wässrigen Lösungen. Hierbei tritt jedoch der Ionenaustausch in den Vordergrund, der je nach Charakter des verwendeten Aluminiumoxyd-Präparates verschieden verläuft. In diesem Sinne werden basische Farbstoffe bevorzugt von basischem (natriumhaltigem), saure Farbstoffe bevorzugt von saurem (Cl-haltigem) Aluminiumoxyd sorbiert[1]. Diese Erscheinungen lassen sich zur Charakterisierung und Standardisierung von Aluminiumoxyd-Präparaten benutzen[2]. Neutrales Oxyd verhält sich jedoch gegenüber diesen Farbstoffen in wässriger Lösung indifferent[3].

Saures Aluminiumoxyd nimmt z.B. den sauren Farbstoff Orange GG (Cassella) auf[2]. War dasselbe durch Behandlung mit überschüssiger Salzsäure erhalten worden, ist die Aufnahme des Farbstoffes eine maximale. Wenn man aber neutrales (eventuell auch basisches) Aluminiumoxyd nur mit einer beschränkten Menge Säure behandelt, indem man über eine Säule eine geringe Menge Säure enthaltende Lösung laufen lässt, dann wird nur soviel Orange adsorbiert, wie der angewandten Menge Säure entspricht[4]. Man kann, nachdem der überschüssige Farbstoff mit Wasser ausgewaschen ist, durch Eluieren des festgehaltenen Orange GG mit Natronlauge und Messen der erhaltenen Lösung in einem Kolorimeter mit Hilfe einer Eichkurve den Effekt zur Bestimmung von Säuren, auch in Spuren, verwenden. Bestimmt wurde nach dieser Methode Kohlensäure, Salzsäure, Schwefelsäure, Essigsäure und Stearinsäure.

Die Untersuchungen wurden nunmehr auf einige Dicarbonsäuren, Hydroxymono-, -di- bzw. -tricarbonsäuren ausgedehnt. Jeweils 200 mg der Säure wurden in 20 ml Wasser gelöst und auf eine Säule aus Aluminiumoxyd Woelm neutral von 10.3 mm Durchmesser und 22 cm Länge gegeben. Nach Entwicklung mit 25 ml einer etwa 0.01 *M* Lösung von Orange GG (gereinigt[*]) wurde der überschüssige Farbstoff erst mit 10 ml, dann nach Einsickern mit 25 ml destilliertem Wasser ausgewaschen. In Tabelle I sind die beobachteten Zonenlängen zusammengestellt, die vom oberen Rand der Säule an gerechnet werden. Bei Tartronsäure, Weinsäure, Citronensäure und Äpfelsäure ist die ganze Säule farblos.

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[*] Das früher[4] verwendete "Orange GG konz. bes. rein" von Cassella wird nicht mehr geliefert. Das stattdessen verfügbare "Orange GG" wird gereinigt, indem man eine Lösung von 5 g Farbstoff in 1 l dest. Wasser zuerst durch eine Säule von 25 g Aluminiumoxyd Woelm sauer, dann durch eine Säule von 260 g Aluminiumoxyd Woelm neutral laufen lässt und das bei beiden Säulen zuerst anfallende farblose Filtrat verwirft. Die so erhaltene gereinigte Lösung ist etwas weniger als 1/100 *M*.

TABELLE I

| | |
|---|---|
| Glykolsäure | 4.5 cm orange |
| Milchsäure | 7.0 cm orange |
| Salicylsäure | 6.5 cm orange |
| Tropasäure | 4.0 cm orange |
| Bernsteinsäure | 12.0 cm orange |
| Malonsäure | 2.0 cm farblos |
| und | 20.0 cm orange |
| Tartronsäure | 22.0 cm farblos |
| Weinsäure | 22.0 cm farblos |
| Citronensäure | 22.0 cm farblos |
| Äpfelsäure | 22.0 cm farblos |

Wie man der Tabelle I entnehmen kann, steht das Verhalten der Hydroxymonocarbonsäuren und Bernsteinsäure in Übereinstimmung mit den früher[4] mitgeteilten Befunden, nach denen die durch das Aluminiumoxyd fixierten Säuren gegen den Farbstoff Orange ausgetauscht werden. Die Länge der Zonen ist davon abhängig, wie stark die betr. Säure vom Aluminiumoxyd festgehalten wird und wie stark das ausgetauschte Orange auf der Säule haftet. Malonsäure zeigt in gewisser Weise einen Übergang zu den untersuchten mehrbasischen Hydroxycarbonsäuren Tartronsäure, Weinsäure, Citronensäure und Äpfelsäure, die ein ganz anderes Verhalten zeigen. Nach dem Entwickeln mit Orange-Lösung und Nachwaschen mit Wasser bleibt keine gefärbte Zone zurück, die ganze Säule ist farblos. Diese Befunde lassen zwei Möglichkeiten offen: entweder werden die Säuren nicht adsorbiert, dann kann natürlich auch kein Austausch gegen Orange stattfinden, oder die Säuren werden zwar festgehalten, werden aber nicht gegen Orange ausgetauscht.

Eine eindeutige Erklärung kann man finden, wenn man sich bei der Chromatographie des sauren Aluminiumoxyds bedient. Hierbei wurden wieder 200 mg der betr. Säure in 20 ml Wasser auf eine Säule aus Aluminiumoxyd Woelm sauer von denselben Ausmassen gegeben, mit 25 ml einer etwa 0.01 *M* gereinigten Orange-Lösung entwickelt und mit 10 + 25 ml Wasser ausgewaschen. Die Ergebnisse sind in Tabelle II enthalten, in der die Zonen wieder vom oberen Rand der Säule an gerechnet werden.

Die Fixierung der Bernsteinsäure findet bei dem sauren Aluminiumoxyd durch Austausch gegen die Cl-haltigen sauren Zentren statt, wobei dann die Säuren wieder weiter gegen Orange ausgetauscht werden. Diese so erhaltene Zone gibt sich durch die Farbe hell-orange zu erkennen, bei der anschliessenden dunkel-orangen Zone handelt es sich um den direkten Austausch der noch vorhandenen salzsauren Zentren

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TABELLE II

| | | | |
|---|---|---|---|
| Bernsteinsäure | | 20.0 cm hell-orange | 1.0 cm dunkel-orange |
| Malonsäure | 1.0 cm farblos | 19.5 cm hell-orange | |
| Tartronsäure | 12.0 cm farblos | | 3.0 cm dunkel-orange |
| Weinsäure | 8.0 cm farblos | 3.0 cm hell-orange | 2.5 cm dunkel-orange |
| Citronensäure | 10.0 cm farblos | | 5.0 cm dunkel-orange |
| Äpfelsäure | 9.0 cm farblos | 4.0 cm hell-orange | 3.0 cm dunkel-orange |

gegen den Farbstoff. Während die Länge der hellen Zone von der Art und Menge der angewandten Säure abhängt, wird die Länge der dunklen von der Menge des angewandten Farbstoffes bestimmt. Bei den mehrbasischen Oxysäuren sehen wir wieder eine farblose Zone. Dieser Effekt kann nur dadurch zustande gekommen sein, dass die Säuren die sauren Zentren durch Austausch verdrängt haben und dann aber nicht gegen Orange ausgetauscht werden. Malonsäure nimmt auch hier wieder eine Zwischenstellung ein.

Auch beim neutralen Aluminiumoxyd lässt sich leicht nachweisen, dass die farblose Zone nicht leer ist, sondern tatsächlich die aufgegebene Säure enthält. Werden zum Beispiel unter den Bedingungen der Tabelle I 200 mg Citronensäure auf die neutrale Säule gegeben und dann vor der Entwicklung mit der Orange-Lösung und dem Auswaschen mit Wasser noch 25 ml einer *N*/1000 Essigsäure nachgegeben, ist nicht wie in Tabelle I die ganze Säule farblos, sondern unter einer farblosen Zone von 14 cm Länge ist die Säule bis zum unteren Ende hell-orange gefärbt: Die Essigsäure wird also *unter* der Citronensäure festgehalten. Wiederholt man denselben Versuch, behandelt aber die Säule vor der Aufgabe der Essigsäure erst mit *N*/10 Natronlauge und dann gründlich mit Wasser, erscheint am oberen Anfang der Säule eine hell-orange Zone. An dieser Stelle muss in Übereinstimmung mit früheren Befunden[4] die Essigsäure sitzen, nachdem die Citronensäure mit Natronlauge eluiert worden war.

Die Erklärung für die beobachteten farblosen Zonen dürfte darin zu finden sein, dass infolge der Affinität der Hydroxylgruppe zum Aluminiumoxyd sich ein Chelat bildet und dadurch die mehrbasischen Hydroxysäuren im Gegensatz zu anderen Säuren so fest an Aluminiumoxyd gebunden sind, dass ein Austausch gegen Orange nicht mehr stattfinden kann. Es ist ein ähnliches Verhalten wie bei Glutaminsäure und Asparaginsäure, die nach Austauschadsorption an saurem Aluminiumoxyd nicht durch den Farbstoff Bromthymolblau, der sonst leicht aufgenommen wird, verdrängt werden[5].

Interessanterweise zeigt das eben geschilderte Verhalten der untersuchten Hydroxysäuren an neutralem Aluminiumoxyd auch noch eine andere Verbindungsklasse, nämlich Phosphorsäureester von mehrwertigen Alkoholen. Unter den Bedingungen der Tabellen I und II, jedoch unter Anwendung der 200 mg Phosphorsäure äquivalenten Mengen und nach Einstellung mit Salzsäure auf pH 1.5, da Salze verwandt wurden, war bei Glycerophosphat, Glucosephosphat und Glykolphosphat die ganze Säule farblos.

Das Auftreten einer farblosen Zone bei den Phosphorsäureestern ist offenbar wieder an die Gegenwart einer Hydroxylgruppe gebunden, wie sich oben bei den Carbonsäuren aus dem unterschiedlichen Verhalten von Malonsäure bzw. Bernsteinsäure einerseits und Tartronsäure bzw. Weinsäure andererseits ergab. Phosphorsäure selbst zeigt die Erscheinung der farblosen Zone nur zum Teil und entspricht in diesem Verhalten etwa der Malonsäure (Tabelle I), ebenso Propylphosphat (Herstellung nach[6]).

Nach BROCKMANN[7] ist die Adsorptionsaffinität von den funktionellen Gruppen abhängig und fällt in der Reihe: COOH, OH, $NH_2$, CO, OAlk. Ein Phosphorsäureester, der anstelle einer freien OH-Gruppe eine freie COOH-Gruppe enthält, muss also erst recht fest an Aluminiumoxyd gebunden sein und nicht durch Orange ersetzt werden, was bei der Untersuchung von Brenztraubensäurephosphat[8] und Ketoglutarsäurephosphat* durch Beobachtung von farblosen Zonen an neutralem Aluminiumoxyd bestätigt wurde.

Bei gleichem Verhalten an neutralem Aluminiumoxyd beobachtet man jedoch bei den Phosphorsäureestern an saurem Aluminiumoxyd im Gegensatz zu den Hydroxysäuren wie Citronensäure nicht eine vollständig farblose Säule, wie Tabelle III zeigt. Die Ergebnisse wurden unter den bisher üblichen Bedingungen gewonnen, jedoch wurden 360 mg Natrium-glycerophosphat bzw. die äquivalente Menge an Calcium-glucosephosphat angewendet, unter Einstellung des pH der Lösung in 20 ml Wasser auf 1.5. Zum Vergleich wurde das Verhalten von Phosphorsäure untersucht (200 mg).

TABELLE III

| | | | |
|---|---|---|---|
| Glycerophosphat | 3.5 cm farblos | 5.5 cm hell-orange | 2.0 cm dunkel-orange |
| Glucosephosphat | 4.5 cm farblos | 9.0 cm hell-orange | 2.5 cm dunkel-orange |
| Phosphorsäure | 2.5 cm farblos | 4.5 cm hell-orange | 2.5 cm dunkel-orange |

Zwischen der Phosphorsäure und ihren Estern besteht nur ein quantitativer Unterschied. Diese Stoffe zusammen stehen in ihrem Verhalten an saurem Aluminiumoxyd zwischen den Säuren vom Typus der Glykolsäure bzw. Bernsteinsäure und den Säuren vom Typus der Citronensäure, werden also weniger fest gebunden als letztere.

Durch die fehlende Austauschfähigkeit gegenüber Orange lassen sich also bestimmte Hydroxycarbonsäuren und Phosphorsäureester in ihrem Gemisch mit anderen Säuren anhand ihres Chromatogramms erkennen bzw. lassen sich markieren und können dann mit Natronlauge eluiert werden.

Während sich die untersuchten Hydroxy-di- bzw. -tricarbonsäuren an Aluminiumoxyd als starke, die untersuchten Phosphorsäureester als schwächere Chelatbildner erweisen, verhalten die sich sonst als Chelatbildner bekannten Säuren, wie Nitrilotriessigsäure und Aethylendiamintetraessigsäure anders. Das Chromatogramm der Nitrilotriessigsäure entspricht etwa dem der Glykolsäure, das der Aethylendiamintetraessigsäure ist ähnlich, jedoch ist bei beiden Oxyden am oberen Ende eine schmale farblose Zone erkennbar.

---

* Diese Verbindung liess sich nach der Methode herstellen, die bei Brenztraubensäure beschrieben ist.

## ZUSAMMENFASSUNG

Während an Aluminiumoxyd fixierte Säuren normalerweise gegen den Farbstoff Orange GG ausgetauscht werden, findet dieser Austausch bei gewissen Hydroxy-di- bzw. -tricarbonsäuren und hydroxylhaltigen Phosphorsäureestern nicht statt. Eine Erklärung für dieses Verhalten dürfte in der infolge Chelatbildung auftretenden festeren Bindung zu finden sein.

## SUMMARY

Normally, acids adsorbed on aluminium oxide can be replaced by the dye Orange GG. However, in the case of certain hydroxy-dicarboxylic and -tricarboxylic acids, and phosphoric esters containing hydroxyl groups, this exchange does not occur. A possible explanation for this behaviour could be sought in stronger adsorption due to chelate formation.

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# ADSORPTION PAPER CHROMATOGRAPHY OF INORGANIC ANIONS IN ACETATE BUFFERS

E. PLUCHET AND M. LEDERER
*Institut du Radium, Laboratoire Curie, Paris (France)*

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Separations of inorganic substances by paper chromatography with aqueous solvents have been investigated by various authors since the early work of SCHÖNBEIN and GOPPELSROEDER. The movement of cations depends largely on the pH of the developing solution as has been summarised by PICKERING[1] and HAYEK[2]. Separations of cations that can be utilised in analytical chemistry are rare[3] unless the substances to be separated are carrier-free tracers[4,5].

MILIČEVIĆ[6] investigated not only cations but also anions and LEDERER AND WARD[7] showed by paper chromatography with *N* KCl that anions move essentially near the liquid front, thus ruling out adsorption on the paper in the interpretation of paper electrophoretic results. RIPAN *et al.*[8] have shown recently that polyanions, namely molybdate and its heteropolyacids and even dichromate, are retained on the paper when developed with saturated KCl solution and that this adsorption depends on the pH value of the solvent. No adsorption was recorded in alkaline solvents. These workers utilise their results in explaining problems of complex chemistry but do not concern themselves with the mechanism of the adsorption.

In recent years paper chromatography with aqueous solvents is also being used extensively for the separation of organic substances. To mention a few examples: phenols by BOSCOTT *et al.*[9] and KEITH *et al.*[10], alkaloids by DE MOERLOOSE[11] and CASINOVI *et al.*[12], anthoxanthins and anthocyanins by GAGE *et al.*[13].

The most useful solvents for organic substances were acetate buffers or aqueous solutions of acetic acid.

Since so far no general study dealing with inorganic anions has been reported, it was decided to carry out an investigation of these anions, as hydrolysis and cation exchange, which seem to govern the behaviour of cations, does not occur with them. It was thus hoped that further information on the mechanism of "adsorption" paper chromatography could be obtained.

## EXPERIMENTAL

Several inorganic anions (as their alkali salts) were chromatographed in an aqueous solution containing 10% sodium acetate and 10% acetic acid on Whatman No. 3 MM paper, as these conditions seem to be the most favourable for numerous separations of phenols and alkaloids. As shown in Table I, quite a number of anions are retained by the paper and move slower than the liquid front.

TABLE I

$R_F$ VALUES OF INORGANIC ANIONS

Solvent: 10 g sodium acetate and 10 ml glacial acetic acid made up to 100 ml with distilled water. Paper: Whatman No. 3 MM. Method: ascending development (at about 20°).

| *Anion* | *$R_F$ value* | *Anion* | *$R_F$ value* |
|---|---|---|---|
| $WO_4^-$ | 0.95 | $AsO_4^{-3}$ | 0.98 |
| $CrO_4^{-2}$ | 0.64 | $AsO_3^{-3}$ | 0.86 |
| $MoO_4^{-2}$ | 0.66 + comet | $Cl^-$ | 0.87 |
| $SeO_3^{-2}$ | 0.87 | $Br^-$ | 0.86 |
| $TeO_3^{-2}$ | 0.66 | $I^-$ | 0.83 |
| $VO_3^-$ | 0.54 | $CNS^-$ | 0.77 |
| $TeO_4^{-2}$ | 0.89 | Ferrocyanide | 1.0 |
| $BO_3^-$ | 0.87 | Ferricyanide | 1.0 |
| | | $Co(NO_2)_6^{-3}$ | 1.0 |
| | | $PO_4^{-3}$ | 1.0 |

The acids that are unstable in acetic acid such as $NO_2^-$ or $BrO_3^-$ could, of course, not be chromatographed. In aqueous 10% sodium acetate the following $R_F$ values were observed for the halogen oxyacids: $BrO_3^- = 0.91$; $IO_3^- = 0.84$ and $IO_4^- = 0.83$ (with a comet to 0).

From Table I it is already obvious that there is absolutely no relationship between the movement and the charge of the anion. Tri- and tetravalent anions seem to move much faster as a rule than mono- and divalent ones.

Several cations were also chromatographed in the same solvent as in Table I and their $R_F$ values are given in Table II.

TABLE II

$R_F$ VALUES OF SOME CATIONS

(Conditions as in Table I)

| *Cation* | *$R_F$ value* | *Cation* | *$R_F$ value* |
|---|---|---|---|
| $Co^{+2}$ | 1.0 | $[CoNO_2(NH_3)_5]^{+2}$ | 0.95 |
| $Ni^{+2}$ | 1.0 | $[Co(NO_2)_2(NH_3)_4]^+$ | 0.87 |
| $Cu^{+2}$ | 1.0 | $[Co(NO_2)_3(NH_3)_3]^0$ | 0.77 |
| $UO_2^{+2}$ | 1.0 | | |

The series of cobaltammines shows that also for the cations the "adsorption" does not follow the ionic charge of the complex as has also been shown previously with other aqueous solvents[14].

It was thus considered that some non-ionic adsorption process is responsible for the different movement of various anions and the following experiments were carried out to investigate its properties.

### *Variation of the $R_F$ value with the amount chromatographed*

One way of examining whether the adsorption isotherm is linear is by chromatographing varying amounts. As shown in Fig. 1 there is no appreciable change for the $R_F$ value of $CrO_4^{-2}$ when 10 $\mu$l of solutions containing from 0.5 % to 3 % of $K_2CrO_4$ are chromatographed side by side on the same sheet. For molybdate the change in $R_F$ value is greater (Fig. 2); however the behaviour of molybdate in acid solution is governed by polymerisation equilibria in addition to the adsorption equilibrium. Hence the results with molybdate serve only to show that with certain ions the $R_F$ value may be found to vary with the concentration.

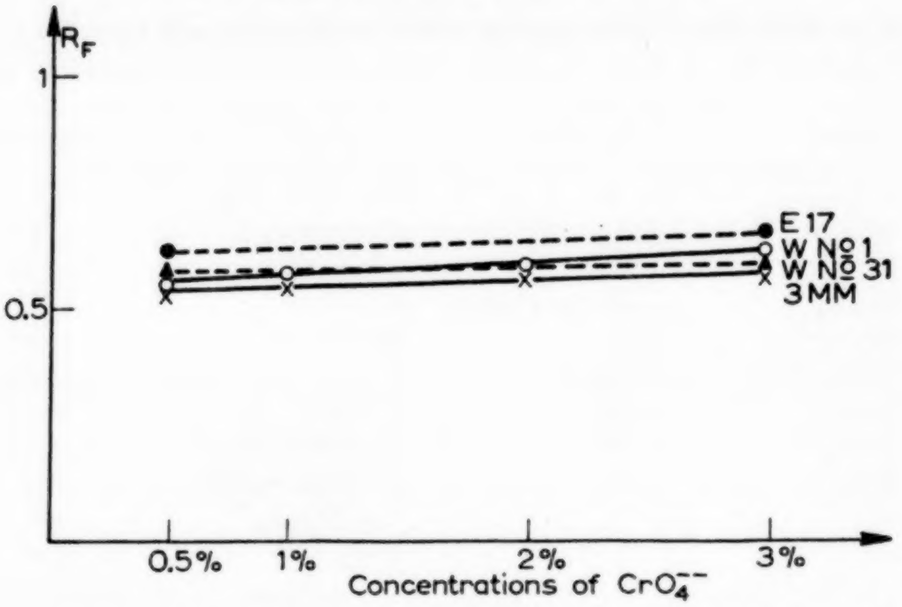

Fig. 1. The $R_F$ values of chromate solutions of varying concentration (0.5 % to 3 %) are plotted for four different brands of Whatman paper (No. 1, No. 31, No. 3 MM and E 17). Solvent: 10 % acetic acid + 10 % sodium acetate.

### *The influence of the solvent to paper ratio on the $R_F$ value*

If an adsorption mechanism is to be ascribed to the retention of anions then the $R_F$ value should vary with the solvent–paper ratio, which would be identical to the factor $A_L/A_S$ of the equation of MARTIN[15]. The solvent–paper ratios were determined for four papers of different thickness by weighing the dry paper and then the developed chromatogram and deducing the liquid weight from that. The results obtained, which are in good agreement with those of other workers[16], are given in Table III.

TABLE III

| *Paper* | *Liquid–paper ratios* |
|---|---|
| Whatman No. 1 | 1.03; 1.01 |
| Whatman No. 3 MM | 1.32 |
| Whatman E 17 | 1.7; 1.69 |
| Whatman No. 31 | 1.41; 1.3 |

The order of the increasing $R_F$ values due to the solvent–paper ratio should thus be: Whatman No. 1 (lowest $R_F$) No. 3 MM, No. 31, E 17 (highest $R_F$). However, as shown in Figs. 1 and 2, this is certainly not the case.

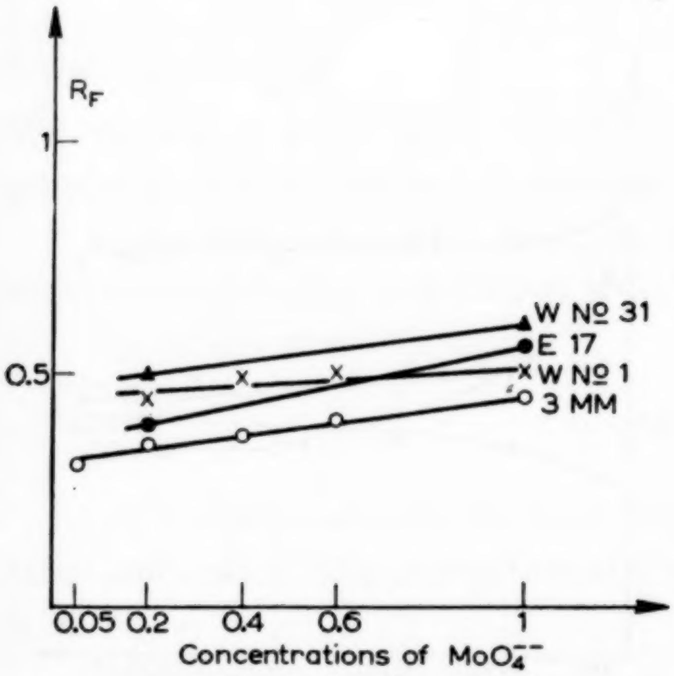

Fig. 2. The $R_F$ values of ammonium molybdate in concentrations from 0.05 % to 1 % on Whatman No. 1, No. 31, No. 3 MM and E 17 papers. Solvent: 10 % acetic acid + 10 % sodium acetate.

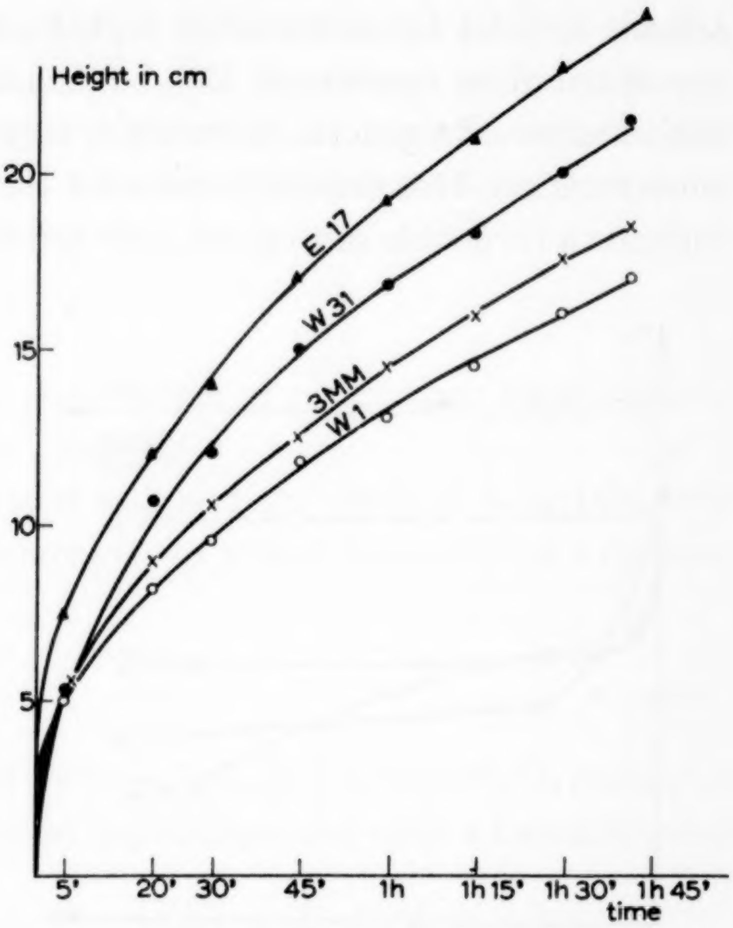

Fig. 3. Liquid movement in ascending development plotted against time on Whatman No. 1, No. 31, No. 3 MM and E 17 papers.

The speed of development should also be a factor, since for a fast liquid flow with a slow equilibrium rate higher $R_F$ values might be obtained than with a liquid flow permitting attainment of equilibrium between the cellulose surface and the solvent. As can be seen from Fig. 3 the speed of development follows the order of the solvent–paper ratios. Thus both factors seem either negligible or overruled by another property of the paper.

### *Effect of pH on the $R_F$ value*

Fig. 4 shows the variation of the $R_F$ values of selenite, chromate, molybdate and metavanadate with the pH value. The pH value was varied by adding increasing amounts of acetic acid to 10 % sodium acetate. Whatman No. 3 MM paper was used only. The "true" anions selenite and chromate do not change their $R_F$ values from pH 5.8 to 4.2, vanadate and molybdate, which undergo polymerisation in this pH range, do vary somewhat.

### *Effect of salt concentration on the $R_F$ value*

We tried to measure the effect of salt concentration without change of the pH by developing with various dilutions of mixtures of sodium acetate and acetic acid at constant ratio (1:1). There is a considerable change for $CrO_4^{-2}$, $MoO_4^{-2}$ and $VO_3^-$ but little change for $SeO_3^{-2}$ as shown in Fig. 5. The small change in $R_F$ value for the selenite may be due to its rather high $R_F$ which does not easily register differences in the distribution coefficient. Molybdate increases its $R_F$ with an increase in the salt concentration. In general, however, it seems that $R_F$ values are depressed by the ionic concentration. This definitely rules out the possibility of anion exchange and strongly suggests a reversible salting-out (precipitation-like) process. It must not be forgotten, however, that in increasing the solute of the developing solvent its water content is considerably decreased and a solution of 20 % sodium acetate and 20 % acetic acid may have dielectric properties which are very different from more dilute solutions.

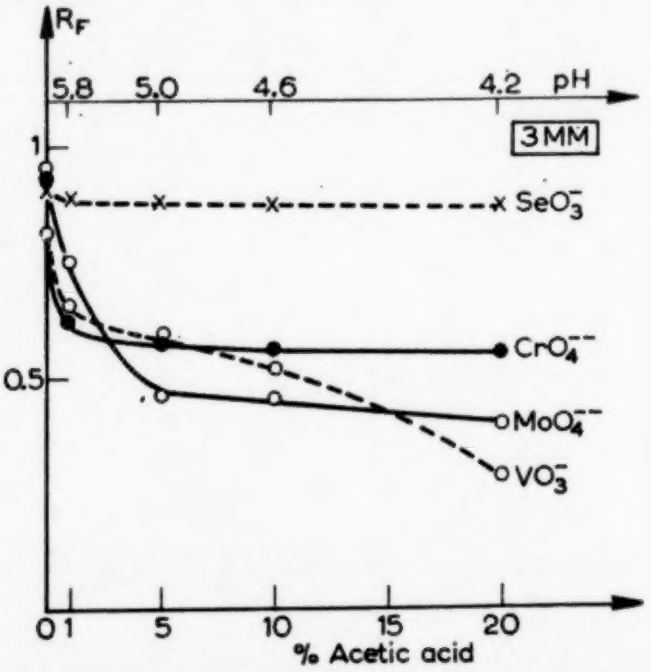

Fig. 4. Variation of $R_F$ values of selenite, chromate, molybdate and vanadate with pH. Varying amounts of acetic acid (0, 1, 5, 10 and 20%) were added to 10% sodium acetate. The pH values indicated above were measured with a glass electrode. Paper: Whatman No. 3 MM.

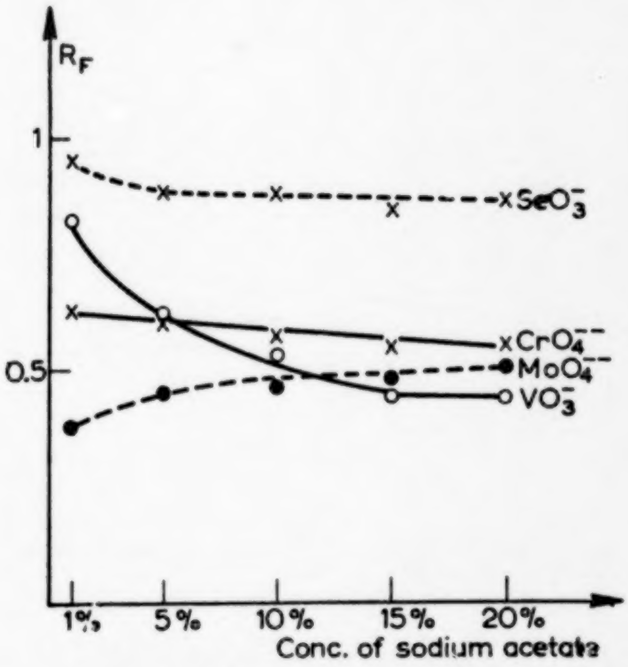

Fig. 5. Variation of $R_F$ values with increase in salt concentration. Equal quantities of sodium acetate and acetic acid (1, 5, 10, 15 and 20%) in increasing amounts are used as solvent. Paper: Whatman No. 3 MM.

### *The effect of changing the cation of the salt in the developing solution*

We substituted Li and K for Na acetate in the solvent which gave as shown in Table IV a higher $R_F$ value for the solvent containing Li and a lower one for that containing K in all cases. This again suggests strongly that the mechanism is one similar to a salting-out process. Results with aluminium acetate and magnesium acetate are

TABLE IV

$R_F$ VALUES OF ANIONS IN ACETATE BUFFERS WITH DIFFERENT CATIONS

| *Solvent* | $MoO_4^{-2}$ | $CrO_4^{-2}$ | $TeO_3^{-2}$ | $SeO_3^{-2}$ | $VO_3^-$ |
|---|---|---|---|---|---|
| Lithium acetate + acetic acid | 0.45 | 0.59 | 0.59 | 0.88 | 0.61 |
| Sodium acetate + acetic acid | 0.44 | 0.56 | 0.61 | 0.86 | 0.47 |
| Potassium acetate + acetic acid | 0.40 | 0.54 | 0.57 | 0.85 | 0.26 |

inconclusive as the pH value varies with the cation. Substitution of NaCl or $Na_2SO_4$ for part of the sodium acetate in the buffer did not give any change in $R_F$ values.

The amounts of Li and K acetate were equivalent to 10 g of Na acetate. The acetate was added to 10 ml glacial acetic acid and the volume made up to 100 ml with distilled water.

### *Some analytical separations*

We considered it necessary to confirm the possibilities of separations by chromatographing several mixtures (conditions as in Table I):

(*i*) *Vanadate–tungstate*. Mixtures separated well and the spots of tungstate were detected with $SnCl_2$ + KCNS in HCl. Vanadate gives a yellow spot without a reagent.

$R_F$ values in mixtures:

| | | |
|---|---|---|
| V | 0.49 | 0.49 |
| W | 0.92 | 0.94 |

(*ii*) *Tellurite–selenite*. Both are detected with $SnCl_2$ in HCl. If tellurite is present in large amounts it may precipitate at the point of application and yield a forward comet from $R_F = 0$.

$R_F$ values in mixtures:

| | | |
|---|---|---|
| $TeO_3^{-2}$ | 0.66 | 0.66 |
| $SeO_3^{-2}$ | 0.87 | 0.88 |

(*iii*) *Arsenite–arsenate*. Detection is best carried out by dipping in a solution of silver nitrate.

$R_F$ values in mixtures:

| | | |
|---|---|---|
| As(III) | 0.86 | 0.86 |
| As(V) | 0.96 | 0.96 |

## DISCUSSION AND CONCLUSION

Our results with the relatively few anions investigated suggest the following conclusions for chromatography on cellulose with acetate buffers:

1. Both adsorption of anions and the change in $R_F$ values with varying ionic strength seem to exclude the possibility of anion exchange.

2. No relation between the solvent–paper ratio and the $R_F$ value could be observed nor was there any correlation between the solvent speed and the $R_F$ value. Thus an adsorption mechanism analogous to the partition mechanism of partition chromatography seems to be unlikely.

3. The influence of the cation of the acetate buffer and of the ionic strength seem to suggest a mechanism similar to salting-out or precipitation.

4. Among the twenty odd inorganic acids stable in acetate buffer only those with a tendency to polymerisation namely $VO_3^-$, $MoO_4^{-2}$ and $CrO_4^{-2}$ are considerably retained by the paper as has already been observed by RIPAN *et al.*[8]. This observation and the sequence of the cobaltammines suggest that the adsorption is favoured by large molecular size and/or low molecular charge.

## SUMMARY

The adsorption paper chromatography of inorganic anions in acetate buffers was studied and the possible adsorption mechanism discussed. Some analytical separations were recorded.

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# USE OF THE "$\pi$"-ELECTRON INTERACTION FOR SELECTIVE SEPARATION OF SOME QUINOLINE BASES, AND AROMATIC AND HETEROCYCLIC HYDROCARBONS FROM COAL TAR DISTILLATES BY GAS–LIQUID CHROMATOGRAPHY

JAROSLAV JANÁK AND MILAN HŘIVNÁČ

*Laboratory for Gas Analysis, Czechoslovak Academy of Science, Brno (Czechoslovakia)*

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The selective sorption that is achieved when aromatic hydrocarbons are separated on stationary phases of aromatic character, is due to the ability of substances of similar structure to form association compounds. Paraffins may thus be readily separated from aromatics that have the same boiling range, *e.g.* cyclohexane from benzene etc.[1].

The substitution of an alkyl group in the aromatic ring causes steric hindrance of the association of these molecules, because the added group disturbs the regular spacing of the "$\pi$"-electron cloud. As regards the association of substances with similar structure (the one being the stationary phase, the other the compound chromatographed) the steric hindrance caused by the alkyl group has a greater effect on the chromatographic sorption (decrease of sorption) than the polarity due to the deformation of the "$\pi$"-electron arrangement on the nucleus. Thus, for instance, when separating polysubstituted biphenyls[2] an elution order was observed which corresponded mainly to the steric position of the methyl groups. Similarly, it is easier to separate ethylbenzene from *m*- and *p*-xylene than these two isomers from each other[3].

If the stationary phases used are electron acceptors, then not only the sorption of the simple aromatic compounds is increased[4], but for their alkyl derivatives that part of the total sorption ability that is due to the forces causing the deformation of the "$\pi$"-electron cloud, is also increased. The effect increases with increasing electronegativity and uniformity of the acceptor. LANGER *et al.*[5] enhanced this effect by increasing the electronegativity of the carbonyl groups of phthalic acid esters, by introducing halogens on the nucleus. The influence of the deformation of the "$\pi$"-electron cloud is illustrated by the reverse order of elution of *m*- and *p*-xylene from di-*n*-propyl tetrachlorophthalate (see Table III).

Increased electronegativity may also be observed in the case of other electron acceptors, such as $\beta,\beta'$-oxydipropionitrile[6,7]. ORR AND CALLEN[8] (see also[9,10]) used the esters of adipic acid with the same effect and achieved considerable selectivity in the separation of esters of fatty acids with one, two or more double bonds.

The fact that the same sorption effect occurs in the case of aromatic compounds and compounds with double bonds led us to investigate the possibility of using the donor–acceptor effect for the separation of some coal-tar substances containing

## TABLE I

RETENTION DATA FOR QUINOLINE AND HIGHER PYRIDINE BASES AT 200°

| *Compound* | *B.p.* °*C* | *Relative retention volumes* | | |
|---|---|---|---|---|
| | | *Reoplex 400* | *Apiezon L* | *Silicon E 301* |
| Quinoline | 238 | 1.00 | 1.00 | 1.00 |
| Isoquinoline | 243 | 1.18 | 1.14 | 1.15 |
| 2-Methylquinoline | 248 | 1.03 | 1.27 | 1.31 |
| 6-Methylquinoline | 258 | 1.36 | 1.54 | 1.53 |
| 7-Methylquinoline | 252 | 1.41 | 1.68 | 1.60 |
| 8-Methylquinoline | 248 | 0.98 | 1.39 | 1.40 |
| 1-Methylisoquinoline | 255 | 1.39 | 1.66 | — |
| 3-Methylisoquinoline | 251 | 1.19 | 1.57 | — |
| 2,4-Dimethylquinoline | 265 | 1.81 | 2.37 | 2.33 |
| 2,6-Dimethylquinoline | 267 | 1.50 | 2.14 | 2.06 |
| 2,4,6-Trimethylquinoline | 288 | 2.63 | 4.02 | 3.70 |
| 2,4,6-Trimethylpyridine | 170 | 0.14 | 0.23 | 0.037 |
| 2,3,5-Trimethylpyridine | 187 | 0.23 | 0.26 | 0.050 |
| 2,3,4-Trimethylpyridine | 192 | 0.30 | 0.48 | — |
| 2,3,5,6-Tetramethylpyridine | 130/100 mm | 0.38 | 0.51 | — |
| 2-Methyl-5-ethylpyridine | 175 | 0.17 | 0.29 | — |
| 4-Methyl-3-ethylpyridine | 196 | 0.26 | 0.46 | — |
| $V_g^0$ for quinoline | | 183 | 132 | 48 |

## TABLE II

RETENTION DATA FOR SOME AROMATIC AND HETEROCYCLIC HYDROCARBONS AT 200°

| *Compound* | *B.p.* °*C* | *Relative retention volumes* | |
|---|---|---|---|
| | | *Reoplex 400* | *Apiezon L* |
| Quinoline | 238 | 1.00 | 1.00 |
| Naphthalene | 218 | 0.54 | 0.82 |
| 2-Methylnaphthalene | 241 | 0.76 | 1.33 |
| 1-Methylnaphthalene | 243 | 0.87 | 1.46 |
| Indole | 253 | 3.20 | 0.97 |
| 2-Methylindole | 273 | 3.83 | 1.94 |
| 3-Methylindole | 266 | 3.71 | 1.89 |
| 5-Methylindole | 270 | 4.04 | 1.97 |
| 7-Methylindole | 268 | 3.57 | 1.79 |
| Biphenyl | 255 | 1.13 | 1.73 |
| 2,6-Dimethylnaphthalene | 261 | 1.06 | 2.18 |
| 1,6-Dimethylnaphthalene | 262 | 1.15 | 2.35 |
| 2,3-Dimethylnaphthalene | 265 | 1.33 | 2.42 |
| Acenaphthene | 278 | 1.75 | 3.20 |
| Diphenyl oxide | 287 | 2.52 | 3.50 |
| Fluorene | 295 | 3.10 | 4.83 |
| $V_g^0$ for quinoline | | 183 | 132 |

alkyl groups on the aromatic ring or the heteroatom, that cause deformations of the original "π"-electron arrangement.

## EXPERIMENTAL

The measurements were carried out with an apparatus of Griffin & George, London, Type MK II with a millivoltmeter recorder (0–3 mV) of Honeywell-Brown. The column was packed with 20–30 % (w/w) substrate on Celite (Johns-Mansville C 545); hydrogen served as the carrier gas. 5–20 μl samples were injected into the columns of approx. 80 cm length and 6–8 mm internal diameter. Detailed working conditions are reported at the respective places. Reoplex 400 (polyoxyalkylene adipate) was a product of Geigy & Co. Ltd., Manchester. Apiezon L Grease (Edwards High Vacuum Ltd.) and Silicon Elastomer E 301 were supplied by Messrs. Griffin & George, London.

The relative and absolute retention data are given in Tables I and II.

## DISCUSSION

Table III shows the retention data of the characteristic model substances on various stationary liquids acting as electron acceptors.

The quantitative increase of the total selectivity to polar substances (carriers

TABLE III

COMPARISON OF SELECTIVITY OF SOME DONOR–ACCEPTOR TYPE BONDING STATIONARY PHASES

| | | Relative retention volumes | | | | | |
|---|---|---|---|---|---|---|---|
| *Compound* | *B.p. °C* | *Bis(ethyl-n-hexyl) sebacate (ref.[11])** | *Di-n-decyl phthalate (ref.[11])** | *Hexaethylene glycol dimethyl ether (ref.[7])* | *Di-n-propyl tetrachlorophthalate (ref.[5])* | *Polyoxyalkylene adipate (Reoplex 400) (found)* | *β,β'-Oxydipropionitrile (ref.[7])* |
| | | | *Temperature 70°* | | | | |
| Carbon tetrachloride | 76.7 | 0.90 | 0.89 | 0.70 | — | 0.59 | 0.36 |
| *n*-Heptane | 98.4 | 0.89 | 0.78 | 0.29 | — | 0.16 | 0.08 |
| Cyclohexane | 81.4 | 0.72 | 0.64 | 0.30 | — | 0.21 | 0.11 |
| Benzene | 80.1 | 1.00 | 1.00 | 1.00 | — | 1.00 | 1.00 |
| Ethylbenzene | 136.2 | 6.00 | 5.90 | — | — | 3.51 | 2.23 |
| | | | *Temperature 110°* | | | | |
| Benzene | 80.1 | 1.00 | 1.00 | — | 1.00 | 1.00 | 1.00 |
| Ethylbenzene | 136.2 | 4.35 | 4.4 | — | 3.05 | 2.82 | 2.36 |
| *m*-Xylene | 139.2 | 4.6 | 4.5 | — | 3.56 | 3.11 | 2.61 |
| *p*-Xylene | 138.4 | 4.6 | 4.45 | — | 3.72 | 2.96 | |
| *o*-Xylene | 144.5 | 5.50 | 5.25 | — | 4.71 | 4.00 | 3.42 |
| *n*-Decane | 174 | 12.9 | 12.9 | — | 2.91 | 1.22 | 0.29 |
| Separation factors: | | | | | | | |
| *o*-Xylene/ethylbenzene | | 1.265 | 1.195 | — | 1.545 | 1.44 | 1.45 |
| *m*-Xylene/*p*-xylene | | — | — | — | 0.957 | 1.050 | — |

* Interpolated values.

of donors) may be pursued on the decreasing retention ratio of electroneutral compounds or compounds with an insignificant polarisation ability (*n*-decane, *n*-heptane, carbon tetrachloride). The type of forces that cause the total selectivity is, however, not uniform, as can be seen from the separation factors. The greatest effect is obtained with phases whose molecules accept the "$\pi$"-type electrons with only a single type of functional group. Though, for instance, polyoxyalkylene adipate or $\beta,\beta'$-oxydipropionitrile have higher total sorption activity, the participation of the "$\pi$"-electron interaction in these substances is lower due to the decrease of the effect of the –CO– or –CN acceptor-active functional groups in the molecule. For comparison the retention data are given of model substances on hexaethylene glycol dimethyl ether, of which the oxygen of the ether bonds also acts as an acceptor. This compound has a structure that is very similar to the alcoholic part of the adipate molecule.

Similar differences in the sorption selectivity may be expected also for higher aromatic hydrocarbons. In the case of quinoline bases the nitrogen, being a slight internal acceptor, acts mainly as a deformer of the "$\pi$"-electron cloud of the whole molecule. Evidence of this is given by the separation factors calculated from the observed data, which are summarized in Table IVa. A quantitatively higher selective effect may be observed in the case of diphenylene oxide, where the hetero-atom, a strong internal acceptor, causes permanent polarisation of the whole diphenylene oxide molecule. Such a permanent dipole causes the increased sorption (Table IVb). But only if the molecule of the chromatographed substance contains a functional group which acts as a donor of the available electron pair by itself, does the order of the sorption selectivity increase due to the formation of fragmental hydrogen bonds between the pyrrole nucleus and all the functional groups of the stationary phase; an example of such a compound is indole (Table IVc).

TABLE IV

COMPARISON OF SEPARATION FACTORS FOR SOME MODEL COMPOUNDS ON APIEZON L AND REOPLEX 400

(Temperature 200°)

| *Compounds* | *Separation factor* | |
|---|---|---|
| | *Apiezon L* | *Reoplex 400* |
| (a) | | |
| 1-Methylnaphthalene/naphthalene | 1.78 | 1.62 |
| 1-Methylnaphthalene/2-methylnaphthalene | 1.10 | 1.15 |
| 2,3-Dimethylnaphthalene/1,6-dimethylnaphthalene | 1.03 | 1.16 |
| Isoquinoline/quinoline | 1.14 | 1.18 |
| 7-Methylquinoline/8-methylquinoline | 1.22 | 1.44 |
| Fluorene/acenaphthene | 1.51 | 1.77 |
| (b) | | |
| Fluorene/diphenylene oxide | 1.38 | 1.23 |
| (c) | | |
| Indole/quinoline | 0.97 | 3.20 |
| Indole/naphthalene | 1.18 | 5.93 |

In Figs. 1 and 2 examples of the selective separation possibilities are illustrated. The higher polyoxyalkylene adipates, in contrast to most of the substrates that show a similar effect, permit working temperatures above 200°, maintaining at the same time their high sorption selectivity. Therefore, they are suitable stationary phases for the analysis of higher boiling unsaturated compounds (terpenes and other hydrocarbons, alcohols, aldehydes, ketones, etc.) in mixtures with saturated compounds, and preferably for the separation of steric isomers of unsaturated substances.

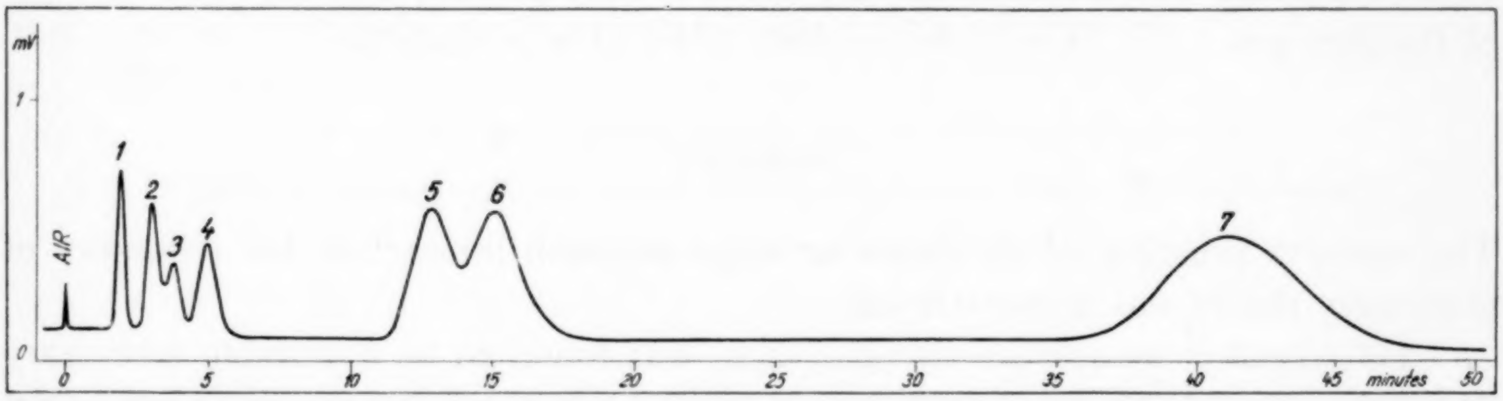

Fig. 1. Gas-chromatographic separation of some coal tar constituents containing nitrogen on Reoplex 400 at 200°. Column 0.7 × 88 cm; flow-rate 0.903 ml $H_2$/sec; inlet pressure 750 mm Hg; outlet pressure 250 mm Hg; 15.5 g packing containing 30% (w/w) substrate on Celite 545. Compounds: (1) 2,4,6-Trimethylpyridine. (2) 2,3,5-Trimethylpyridine. (3) 2,3,4-Trimethylpyridine. (4) 2,3,5,6-Tetramethylpyridine. (5) Quinoline. (6) Isoquinoline. (7) Indole.

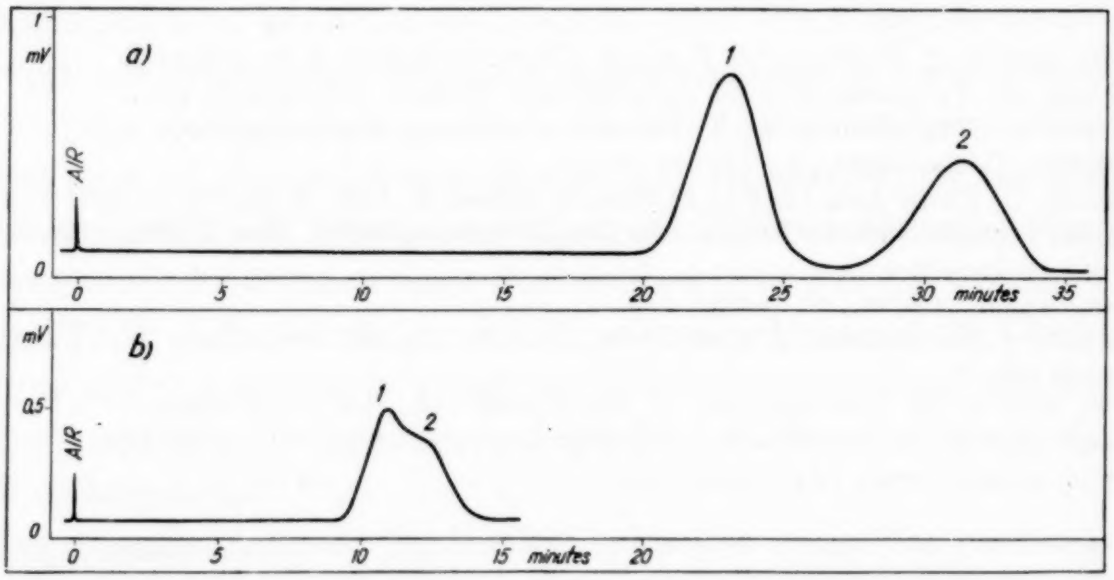

Fig. 2. Comparison of the separation of acenaphthene–diphenylene oxide on Reoplex 400 and on Apiezon L at 200°. (a) Column 0.7 × 88 cm; flow-rate 0.903 ml $H_2$/sec; inlet pressure 750 mm Hg; outlet pressure 250 mm Hg; 15.5 g packing containing 30% (w/w) Reoplex 400 on Celite 545. (b) Column 0.6 × 81 cm; flow-rate 0.833 ml $H_2$/sec; inlet pressure 756 mm Hg; outlet pressure 290 mm Hg; 7.9 g packing containing 20% (w/w) Apiezon L on Celite 545. Compounds: (1) Acenaphthene. (2) Diphenylene oxide.

## ACKNOWLEDGEMENTS

The authors are grateful to the Management of the Chemical Institute of the Lithuanian Academy of Sciences, Riga, U.S.S.R., for kindly providing samples of pure homologues of pyridine and quinoline, further to Dr. R. OBERKOBUSCH of the Gesellschaft für Teerverwertung G.m.b.H., Duisburg-Meiderich, Germany, for kindly providing samples of pure homologues of isoquinoline and indole, and to Dr. W. STUVE of the Margarine-Union Ltd., Hamburg-Bahrenfeld, Germany, for supplying a sample of Reoplex 400.

## SUMMARY

The common principle of the donor–acceptor sorption interaction for a number of stationary phases was demonstrated.

Polyoxyalkylene adipate (Reoplex 400) was found to be a suitable stationary phase for the selective separation of quinoline bases, unsaturated, aromatic and heterocyclic compounds boiling up to 300°. At 200° the retention volumes of model substances were measured and verified by examples of suitable separation of substances from coal tar distillates.

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# GROUND UNGLAZED TILE—A NEW SUPPORT FOR GAS-LIQUID CHROMATOGRAPHY*, **

VÍT LUKEŠ, RADKO KOMERS AND VLASTIMIL HEROUT
*Institute of Chemistry, Czechoslovak Academy of Science, Prague (Czechoslovakia)*

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The preparative separation of the terpene components of essential oils by means of gas–liquid chromatography[1] requires supports of higher qualities than separation for analytical purposes. The material must have great permeability since the separation is carried out in columns of considerable length. Since some terpenes are unstable at higher temperatures, the separation must be performed under mild conditions, *i.e.* under reduced pressure. The choice of the rate of flow of the carrier gas is thus limited by the maximum inlet pressure permissible. One strict requirement is complete chemical inertness of the support. This condition must be observed very closely, particularly in the separation of terpenes sensitive to the catalytic action of metallic ions or those that at elevated temperatures may be affected by the acidic or basic nature of the support itself. Finally, an important feature is the mechanical resistance of the support towards abrasion since dust formed in any of the operations results in irregular streaming of the carrier gas and causes an increase of the pressure gradient in the column and this adversely affects the elution of individual components.

These requirements are not met by any of the common supports such as celite[1], sterchamol[2] or kieselguhr[3]***.

This was already observed by NAVES[4], who also found the customary supports unsuitable for the separation of essential oils. He suggested using classified sea-sand or sodium chloride as inert support. We have now found that finely ground unglazed white tile is an inert support of high quality.

## EXPERIMENTAL AND RESULTS

### *Preparation of the support*

Unglazed tile ("Rako")† was crushed in a ring-roller mill, screened on sieves to the grain size of 0.2–0.3 mm, decanted with water to remove the dust, dried and ignited at 300°.

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[*] Czechoslovak patent application for the use of unglazed tile as a support is pending (PV 6264/58).

[**] Presented at the 6th Meeting of workers in the field of Gas Chromatography, Gottwaldov, June 1959.

[***] Celite (C-545, Johns Manville, London); sterchamol (Sterchamolwerke, Dortmund); kieselguhr SK (Calofrig, Borovany near České Budějovice).

[†] Ceramic Works of Rakovník (National enterprise), Rakovník.

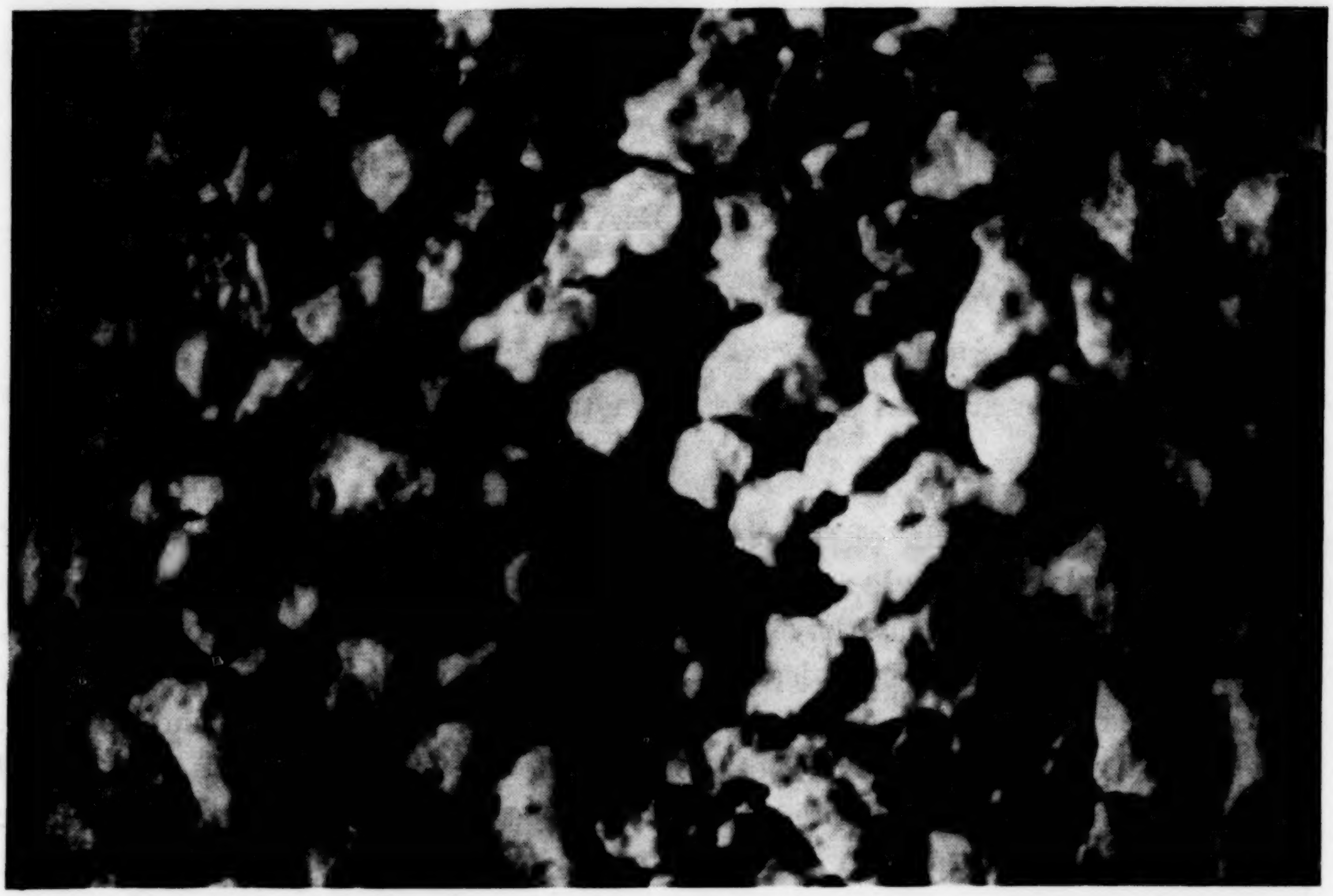

Fig. 1. Microphotograph of the tile support, magnified 30 times.

The support prepared in the manner described is a white material which is mechanically very resistant in comparison with sterchamol or kieselguhr. The individual particles have an irregular shape (Fig. 1). The surface of a fraction with a particle size of 0.2–0.3 mm, measured by the BET[5] method, is 2.2 $m^2/g$.

This tile support was wetted with an ethereal solution of tricresyl phosphate so that the concentration of tricresyl phosphate was 5 %, 7 %, 10 %, 13 % and 18 %, respectively.

### *Efficiency of the support*

To compare the efficiency of this support with that of sterchamol, a simple artificial mixture of monoterpene hydrocarbons ($\alpha$-pinene, myrcene, $\Delta^3$-carene, limonene, and *p*-cymene) was chromatographed, under identical conditions, on sterchamol wetted

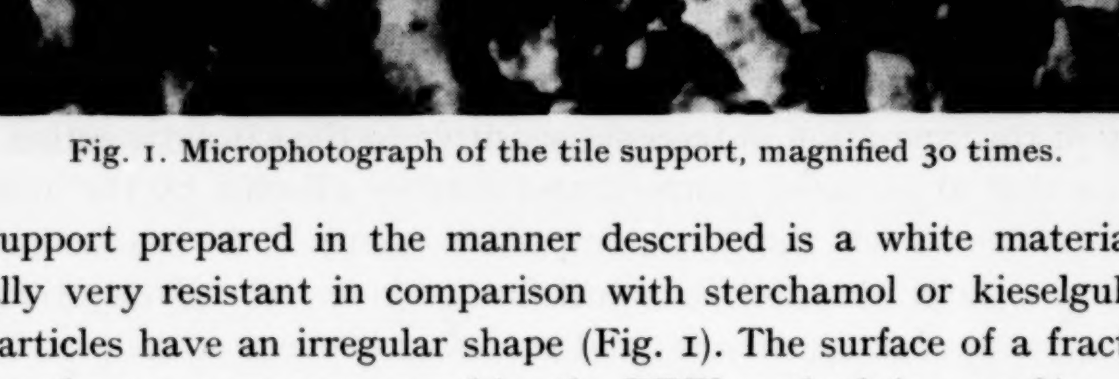

Fig. 2. Chromatogram of a mixture of monoterpene hydrocarbons: (1) $\alpha$-pinene; (2) myrcene; (3) $\Delta^3$-carene; (4) limonene; (5) *p*-cymene. Support: sterchamol; stationary phase: tricresyl phosphate (20 %); temperature: 138°; column length: 240 cm; rate of flow: 19.7 ml $N_2$/min.

with 20% tricresyl phosphate[6] (Fig. 2), and on the tile support containing varying amounts of the stationary phase. As can be seen from Fig. 3, optimum separation was obtained by using a support containing 7% of the stationary phase. Column efficiency is expressed as number of theoretical plates[7] for $\Delta^3$-carene. Column resolution was calculated for myrcene and $\Delta^3$-carene by methods recommended in the literature[8,9].

In order to determine whether the tile support catalyses chemical reactions of the compounds being chromatographed, as is the case with kieselguhr, the following experiment was carried out: 2 ml of pure $\alpha$-pinene was boiled, the vapours were passed through a 5 cm thick layer of tile support heated electrically to 150–160°, and then condensed by a reflux condenser. Three comparative experiments were carried out using unglazed tile, sterchamol and kieselguhr support, respectively. Neither sterchamol nor the tile support caused any noticeable chemical change, whereas kieselguhr produced some $\beta$-pinene as judged by infra-red spectroscopy of the starting and recovered material.

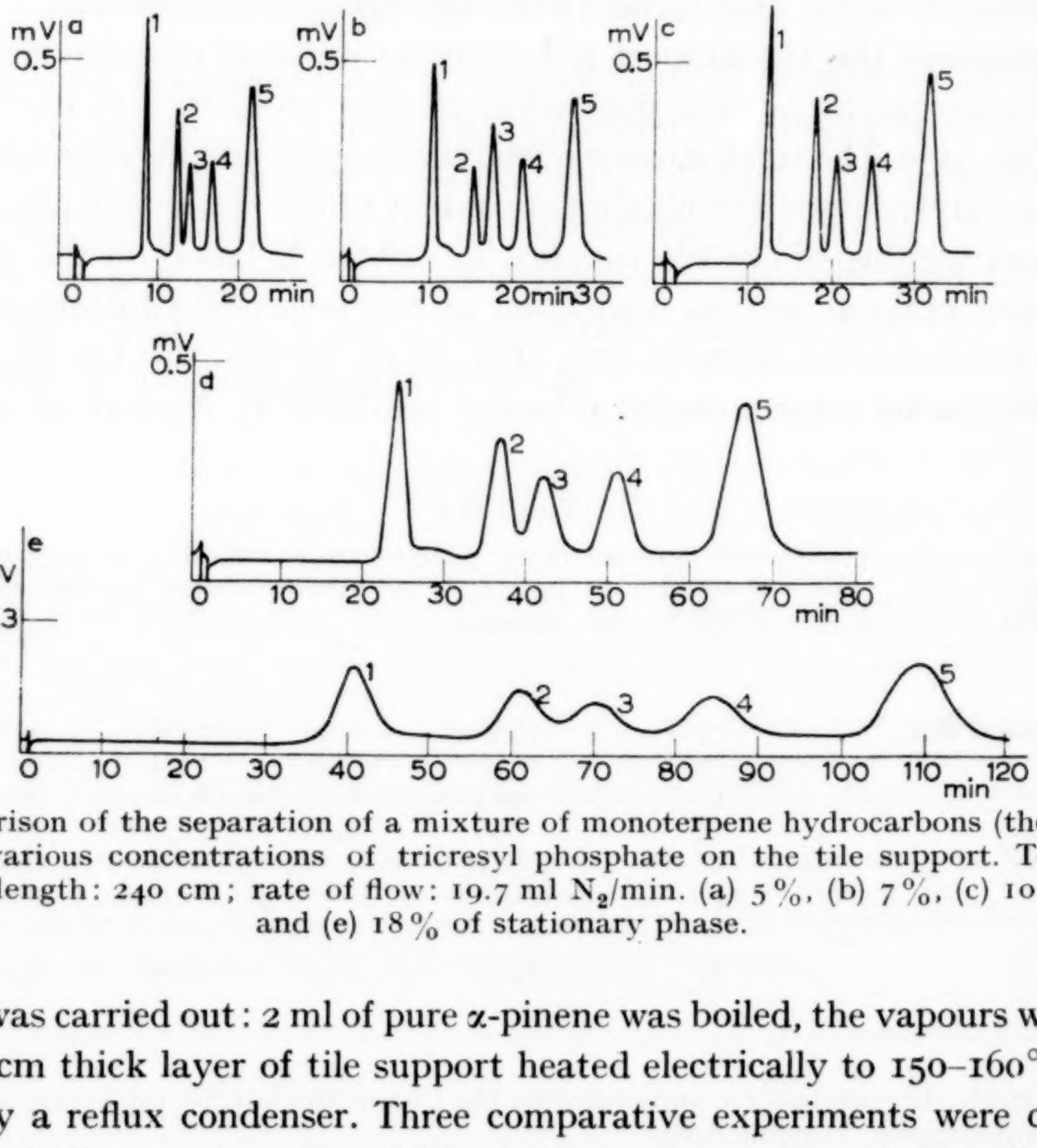

Fig. 3. Comparison of the separation of a mixture of monoterpene hydrocarbons (the same as in Fig. 2) with various concentrations of tricresyl phosphate on the tile support. Temperature: 136°; column length: 240 cm; rate of flow: 19.7 ml $N_2$/min. (a) 5%, (b) 7%, (c) 10%, (d) 13% and (e) 18% of stationary phase.

### *Apparatus and experimental conditions*

All measurements were carried out with the analytical apparatus of Griffin and George (London), type IIA, using a conventional set-up containing a thermal conductivity platinum detector and a millivoltmeter-recorder with the range 0–1 mV. The carrier gas was nitrogen, the temperature of the air-thermostat 136° and the length of the chromatographic column 240 cm.

The preparative separation was carried out with an improvised apparatus, which will be described later. This apparatus had a column of 4.5 m by 17 mm and was capable of separating 0.7 g of a mixture of terpene hydrocarbons.

## DISCUSSION

As compared with currently used supports, the tile support has a considerably greater mechanical resistance. It resists abrasion and does not form fine dust. This property is especially advantageous in the case of long columns, where the permeability is adversely affected by the dust formed from kieselguhr or sterchamol.

The surface of the tile support is less by an order of magnitude than that of sterchamol or kieselguhr, but this does not seem to be prohibitive to its use, as can be seen from Fig. 3a–e. The small surface of the tile support and also the smaller volume of the pores merely lowers the maximum content of the stationary phase. However, the maximum wetting of the tile support, *i.e.* 18 wt.% based on the weight of the support is not optimal for the separation of the terpene hydrocarbons, the most convenient concentration being 7–10% (Fig. 3b,c). In this case the same resolution as on the sterchamol column can be achieved (see Table I). A great advantage of the tile support is further its chemical inertness to unsaturated compounds as was shown by the experiment with α-pinene described in the experimental part.

TABLE I

| *Support* | *Tricresyl phosphate %* | *TP* for $\Delta^3$-carene* | *Column resolution*** | | *Pressure in column, mm Hg* | |
|---|---|---|---|---|---|---|
| | | | *A* | *B* | *inlet* | *outlet* |
| Ground unglazed tile | 5 | 1536 | 1.25 | 0.58 | 446 | 193 |
| | 7 | 2270 | 1.41 | 0.94 | 401 | 207 |
| | 10 | 1025 | 1.12 | 0.82 | 670 | 455 |
| | 13 | 868 | 0.98 | 0.73 | 416 | 210 |
| | 18 | 712 | 0.88 | 0.64 | 671 | 359 |
| Sterchamol | 20 | 1745 | 1.53 | 0.93 | 671 | 621 |

* Calculated by the method recommended by the Committee[7] of the 1st Symposium on Vapour Phase Chromatography, London, 1956.

** A: Calculated by the method recommended by the Committee[8] of the 2nd Symposium on Gas Chromatography, Amsterdam, 1958. B: Calculated by the method of STRUPPE[9].

The high mechanical resistance allows an easy recovery of the tile support by extraction of the stationary phase and ignition of the dried material. The tile support has the further advantage of easy availability, simple recovery and low cost.

In addition to the analysis and preparation of monoterpene hydrocarbons, this support proved to be suitable for the analytical separation of other mixtures such as monoterpene alcohols, monoterpene ketones, esters of dicarboxylic acids[10], stereoisomers of cyclic alcohols[11], etc.

## ACKNOWLEDGEMENTS

The measurement of the surface of the tile support was carried out by Ing. J. Franc (Control and Test Institute, Pardubice-Rybitví), the spectral measurements by Dr. M. Horák in the Department of Physical Chemistry of the Institute of Chemistry of the Czechoslovak Academy of Science. We are indebted to S. Vašíčková, B. Váchová and M. Vašíček for technical assistance.

## SUMMARY

A new support for gas–liquid chromatography made of crushed unglazed white tiles is described. Its surface is 2.2 m$^2$/g, the maximum wettability is 18% for tricresyl phosphate, the optimum amount of the stationary phase for the separation of monoterpene hydrocarbons being 7–10%. This support has no catalytic effects on terpene hydrocarbons, is mechanically very resistant, allows relatively high flow rates, and is easy to recover. Its function was experimentally compared with that of sterchamol using a mixture of monoterpene hydrocarbons.

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# STATISTISCHE AUSWERTUNG DER MESSMETHODEN ZUR QUANTITATIVEN BEARBEITUNG VON REGISTRIERTEN DIFFERENTIALKURVEN IN DER GAS-CHROMATOGRAPHIE*

JAROSLAV JANÁK
*Laboratorium für Gasanalyse, Tschechoslovakische Akademie der Wissenschaften, Brno (Tschechoslowakei)*

(Eingegangen den 17. Juni 1959)

Bei der quantitativen Analyse von gasförmigen und flüchtigen Substanzen mit Hilfe der Gas-chromatographie steht man immer dem Problem gegenüber, auf welche Weise die tatsächliche Menge der im Gemisch enthaltenen Komponenten festgestellt werden soll. Die chromatographischen Methoden, die auf der direkten Messung von Komponenten beruhen (Volum- und Druckmessungen, Titration, Kolorimetrierung, polarographische Messungen u.a.), liefern integrale Angaben. Die meisten in der Gaschromatographie benutzten Detektionsmethoden (Wärmeleitfähigkeitszellen, Mikroflammenbrenner von Scott, Martin's Densitometer, Ionisationsdetektoren u.a.) geben indirekte Angaben.

Mit Hilfe dieser Detektoren erhält man die allgemein bekannten, mehr oder weniger symmetrischen Derivationskurven, deren Form der Gauss'schen Fehlerverteilungskurve gleicht. Den wichtigsten Parametern dieser Kurve gemäss wird dann die Menge der Substanz ausgedrückt. Die bedeutendsten der für diese Berechnungen verwendeten Parameter sind die Flächen und die Höhen der Kurven. In der Literatur[1] und Praxis von heute verwendet man zur Messung dieser Parameter verschiedene Methoden, unter der Voraussetzung, dass sie mit einem Fehler von 1–3% behaftet sind. Um Klarheit in diesen Zustand zu bringen, haben wir ungefähr 100 Analysen mit Hilfe von 7 konstruktiven und 3 automatischen Methoden statistisch verarbeitet[2].

## *Die konstruktiven Methoden*

Die Fläche wird planimetrisch errechnet, durch Wiegen des Papiers mit den unter den Kurven ausgeschnittenen Flächen, durch die Quadratur der Kurve mit Hilfe des Produktes der Höhe und der Breite in der halben Höhe[3], durch Überlagerung eines Dreiecks durch die Tangenten in den Inflexionspunkten der Kurve.

Ausserdem misst man die Höhe im Maximum der Kurve und schliesslich wird auch die Fläche festgestellt, die durch das Produkt der Höhe und der Elutionszeit, bezw. der Entfernung des Maximums vom Anfang des Chromatogramms gegeben ist.

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* Auszug des Vortrages an der Internationalen Messkonferenz in Budapest, November 1958.

Ob sich die verwendete Methode für den gegebenen Zweck eignet, wird einerseits durch den Charakter der Kurve, andererseits durch die konstruktionstechnischen Möglichkeiten der einzelnen Methoden bedingt. Die Form der Kurve spielt beim Planimetrieren keine Rolle, doch ist die Verwendung dieser Methode bei extrem kleinen und grossen Flächen durch die geringe Empfindlichkeit des Planimeters und den bedeutenden persönlichen Messfehler begrenzt.

Beim Wiegen der ausgeschnittenen Flächen ist die Form der Kurve gleichfalls ohne Belang. Die Fehler dieser Methode sind grösstenteils subjektiv—ohne Rücksicht auf die Unhomogenität des Papiers. Sie entstehen dadurch, dass man, besonders bei kleinen Flächen, das Papier gewöhnlich an der äusseren Seite der Linie ausschneidet. Ein grundliegender Nachteil dieser verhältnismässig zeitraubenden Methode ist die totale Vernichtung des Chromatogramms.

Die Methode der Quadratur der Kurve — Höhe mal Breite in halber Höhe[3] — hängt vom Charakter der Kurve ab. Bei unsymmetrischen Zonen werden in die Messung bedeutende Fehler übertragen. Bei kleinen Flächen mit geringer Höhe und beträchtlicher Elutionszeit ist der Parameter der Breite in der halben Höhe schwer zu bestimmen, da die Registriervorrichtung gewöhnlich nicht glatt, sondern in Impulsen (in Stufen) arbeitet. In Bezug auf ihre Durchführung ist die Methode leicht und rasch.

Die Methode des Einzeichnens eines Dreiecks in die Elutionskurve ist für schlanke und hohe Zonen ungeeignet. Es ist manchmal schwierig, die Seiten des Dreiecks zo su führen, dass sie den Inflexionspunkt der Kurve durchschneiden und die Kurve so lang als möglich verfolgen. Diese Methode stellt auf die Durchführung keine hohen Ansprüche, die Höhen sind gut messbar.

Die Methode zur Messung der Fläche, die durch das Produkt der Kurvenhöhe im Maximum und der Elutionszeit, bezw. der Entfernung vom Anfang des Chromatogramms gebildet wird, beruht auf der Messung von ausgeprägten Parametern. Sie ist rasch, hängt aber vom Charakter der Elutionskurven ab. Bei unsymmetrischen Kurven darf diese Methode nicht angewendet werden.

### *Die automatischen Methoden*

Die automatischen Methoden der Flächenintegration unter der Kurve hängen von den subjektiven Fehlern des Beobachters nicht ab, aber auch hier gibt es spezifische und unterschiedliche Fehlerquellen. Wir haben drei grundliegende Integratorentypen überprüft: den mechanischen, elektromechanischen und elektronischen.

Der *mechanische Integrator* (Regula, Praha) stellt das System eines rotierenden Rades und eines Programm-Scheibendaumens dar. Dieser wird durch die Ausgangsspannung des Verstärkers des registrierenden Chromatographen-Millivoltmeters eingestellt. Auf diese Weise bedingt er die mechanische Übertragung der Ausgangsspannung des äquivalenten Teiles der Radumdrehung auf das Getriebe des Numerators. Dieser Typ verursacht Fehler hauptsächlich durch die langsame Reaktion, Reibung und niedrige Impulszahl auf 1 mm² der gemessenen Fläche. Dies kommt vor allem bei kleinen Flächen zum Ausdruck, und zwar in höherem Mass bei sehr flachen Kurven als bei schlanken und hohen. Obzwar dieser Integrator abweichende Konstruktion aufweist, dürfte er in der Praxis dem mechanischen Integrator des Typs "Disk and Ball" (z.B. der Firma Fisher-Gulf Scientific Instruments, u.a.) gleichwertig sein.

Der *elektromechanische Integrator*[4] überträgt die Änderung der verstärkten Spannung vom Detektor des Chromatographen auf die Umdrehungen eines Gleichstrom-Elektrometers, dessen Empfindlichkeit mit Hilfe eines durch einen photoelektrischen Komparator gelenkten Verstärkers erhöht wird. Die Umdrehungszahl der Elektrometerscheibe wird photoelektrisch registriert und mit Hilfe eines modifizierten Telephongesprächzählers errechnet. Die wichtigsten Fehler werden durch den Trägheitswiderstand der Scheibe hervorgerufen, was sich hauptsächlich bei den schlanken Zonen am Anfang des Chromatogramms äussert. In der Praxis steht dieser Typ den Scheibenintegratoren der Firma Instron Eng. Corp. nahe.

Der weiter geprüfte *elektronische Integrator* war ein Analog-Integrator (konstruiert von BÖHM[5]). Die verstärkte Spannung aus dem Detektor wird auf den Eingang geleitet, der durch eine Reaktanzröhre gebildet wird. Diese ist ein Bestandteil des Resonanzkreises eines von zwei Oszillatoren des Generators, der nach dem Schwebungsverfahren arbeitet. Der Ausgang dieses Generators leitet die Impulse an den Numerator und weiter an die automatische Rechenvorrichtung; die Registration und die Nullstellung erfolgt über einen Kippstromkreis. Der Apparat hat praktisch kein Beharrungsvermögen und liefert auf 1 mm² Fläche eine verhältnismässig grosse Anzahl von Impulsen. Dieses Gerät hat bisher auf dem Markt kein Äquivalent.

### *Genauigkeit der Methoden*

Vergleichen wir nun die Genauigkeit aller Methoden auf Grund der bei den Analysen festgestellten Fehler. Die erhaltenen Ergebnisse sind in Tabelle I zusammengefasst.

Man sieht, dass im Bereich der Flächen bis 50 mm² die meisten Methoden einen beträchtlichen Fehler aufweisen, der auf einer Fläche von 25 mm² zwischen 15–25 % relativ schwänkt. Bei Flächen, die kleiner sind als 25 mm², ist mit den grössten Fehlern bei der planimetrischen Methode und beim mechanischen Integrator zu rechnen. Gute Ergebnisse zeigt die Methode der Höhenmessung und der Integration, die nicht durch den Trägheitsfaktor belastet ist.

Im Bereich der Flächen von 51–100 mm² haben die meisten Methoden einen Fehler von 10 % relativ. Nur wiederholtes Planimetrieren, die auf Höhenmessung beruhenden Methoden und die elektrischen Integratorentypen weisen bessere Ergebnisse auf.

Bei grösseren Flächen als 100 mm², was im Grunde genommen die geforderten, zur quantitativen Messung des Chromatogramms geeigneten Flächen sind, liegen die Fehler bereits im Bereich der in der Literatur angegebenen Werte.

Zusammenfassend kann gesagt werden, dass heutzutage gewöhnlich noch nicht mit optimalen Methoden gearbeitet wird. Die besten Ergebnisse für den gesamten Flächenbereich liefert der Analog-Integrator und die Methode der Höhenmessung. Auch einfache Integratorentypen gleichen in ihrer Genauigkeit den allgemein üblichen planimetrischen Methoden und der Quadratur der Kurven, oder übertreffen sie sogar.

TABELLE I

GENAUIGKEIT DER MESSUNG MIT HILFE VON VERSCHIEDENEN METHODEN

| | *Mittlere Fehler*[a] *für Flächen in mm²* | | | |
|---|---|---|---|---|
| | *0–50*<br>*ϕ 25* | *51–100*<br>*ϕ 75* | *101–300*<br>*ϕ 200* | *301–1200*<br>*ϕ 600* |
| *Planimetrieren*[b]: | | | | |
| mm² | 10.0 | 6.84 | 10.0 | 35.0 |
| $d_\phi$ | 40 % | 9.1 % | 5.0 % | 5.8 % |
| *Fünffaches Planimetrieren*: | | | | |
| mm² | 5.75 | 3.84 | 4.61 | 15.6 |
| $d_\phi$ | 23.0 % | 5.1 % | 2.3 % | 2.6 % |
| *Wiegen*[c]: | | | | |
| mm² | 4.37 | 7.53 | 5.50 | 15.2 |
| $d_\phi$ | 17.5 % | 10.0 % | 2.8 % | 2.5 % |
| *Eingezeichnetes Dreieck*: | | | | |
| mm² | 4.18 | 7.14 | 6.8 | 5.6 |
| $d_\phi$ | 16.7 % | 9.5 % | 3.4 % | 0.9 % |
| *Höhe mal Breite in halber Höhe*: | | | | |
| mm² | 4.80 | 9.38 | 8.52 | 14.6 |
| $d_\phi$ | 19.2 % | 12.5 % | 4.3 % | 2.4 % |
| *Höhe mal Elutionszeit mal Faktor*[d]: | | | | |
| mm² | 5.45 | 3.85 | 5.35 | 10.3 |
| $d_\phi$ | 21.8 % | 5.1 % | 2.7 % | 1.7 % |
| *Mechanischer Integrator* (1 Impuls für 5 mm²): | | | | |
| mm² | 9.05[e] | 8.17 | 4.80 | 12.0 |
| $d_\phi$ | 36.2 % | 10.8 % | 2.4 % | 2.0 % |
| mm² | 5.85[f] | | | |
| $d_\phi$ | 23.4 % | | | |
| *Elektromechanischer Integrator* (1 Impuls für 2 mm²): | | | | |
| mm² | 2.50 | 2.25 | 5.40 | 7.80 |
| $d_\phi$ | 10.0 % | 3.0 % | 2.7 % | 1.3 % |
| *Analogintegrator* (8 Impulse für 1 mm²): | | | | |
| mm² | 0.55 | 1.28 | 1.80 | 7.80 |
| $d_\phi$ | 2.2 % | 1.7 % | 0.9 % | 1.3 % |
| *Mittlere Fehler der Höhen in mm für Flächen in mm²*: | | | | |
| mm | 0.60 | 1.23 | 1.43 | 2.80 |
| $d_\phi$ | 4.0 % | 3.5 % | 1.9 % | 1.9 % |

[a] Mittlere Fehler nach der Formel: $\sqrt{\frac{\Sigma\Delta^2}{n-1}}$

[b] Fehler des Polarplanimeters Reiss, DDR, in der optimalen Einstellung für Flächen 100–125 mm²: Rechteckiges Dreieck nach dem Lineal umgelaufen 1.0 %; wiederholte Messung der Fläche unter derselben Kurve 1.6 % (Beobachter A) und 2.3 % (Beobachter B).

[c] Unhomogenität des Papiers 1.25 %.

[d] Der Faktor ist die Zahl, mit welcher das Produkt der Höhe und der Entfernung vom Anfang des Chromatogramms auf die Flächengrösse unter der Elutionskurve in mm² umgerechnet wird.

[e] Fehler für flache Elutionskurven.

[f] Fehler für schlanke Elutionskurven.

## ZUSAMMENFASSUNG

In der Literatur und Praxis von heute verwendet man zur Messung der gas-chromatographischen Kurven verschiedene Methoden unter der Voraussetzung, dass sie mit einem Fehler von 1–3 % behaftet sind. Um Klarheit in diesen Zustand zu bringen, haben wir ungefähr 100 Analysen mit Hilfe von 7 konstruktieven und 3 automatischen Methoden statistisch verarbeitet.

Die konstruktiven Methoden wurden geprüft: Planimetrieren; wiederholtes Planimetrieren; Wiegen des Papiers mit unter den Kurven ausgeschnittenen Flächen; Quadratur der Kurve mit Hilfe des Produktes der Höhe und der Breite in der halben Höhe und mit Hilfe der Überlagerung eines Dreiecks durch Tangenten in den Inflexionspunkten der Kurve; Messung der Höhe der Kurve und Messung der Fläche, Höhe mal Elutionszeit, bezw. mal Entfernung des Maximums vom Anfang des Chromatogramms. Von den automatischen Methoden wurden drei Haupttypen von Integratoren, und zwar ein mechanischer, ein elektromechanischer und ein rein elektronischer Integrator überprüft. Der mechanische Integrator arbeitet auf dem Prinzip eines Systems von rotierendem Rad und Programm-Scheibendaumen. Der elektromechanische Typus überträgt die Änderung der verstärkten Spannung vom Detektor des Chromatographen auf die Umdrehung eines Gleichstrom-Elektrometers; der elektronische Integrator war einer vom Analogtypus.

Es wurden die mittleren Fehler für verschiedene Flächengrössen (0–50 mm², 51–100 mm², 101–300 mm² und 301–1200 mm²) festgestellt. Zusammenfassend kann gesagt werden, dass heutzutage gewöhnlich noch nicht optimale Methoden verwendet werden.

## SUMMARY

In practice various methods are used to measure the curves obtained in gas chromatography, under the assumption that the error in the measurements is 1–3 %. To find out whether this assumption is correct, about 100 analyses were statistically evaluated using 7 geometrical and 3 automatic methods.

The following geometrical methods were tested: planimetry; repeated planimetry; cutting out the areas below the curves and weighing the paper; quadrature of the curve, by means of the product of height and half-width of the peak and by means of a triangle through the tangents at the inflexion points of the curve; measurement of the height of the peak and of the area: height multiplied by elution time, or by the distance of the peak from the starting point of the chromatogram. As regards the automatic methods, three main types of integrators were tested, *viz.* a mechanical, an electromechanical and a completely electronic integrator. The mechanical integrator was based on the principle of a rotating wheel and a timed cam wheel. In the electromechanical model the changes in the amplified output voltage of the detector of the chromatograph were transmitted to a d.c. electrometer; the electronic integrator was of the analogue type.

The mean errors were determined for areas of various sizes (0–50 mm², 51–100 mm², 101–300 mm², and 301–1200 mm²). The results of the investigation lead to the conclusion that the methods currently used are not yet optimal.

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# ZUR PRÄPARATIVEN GAS-CHROMATOGRAPHIE

## ZENTRIFUGALKÜHLER ZUR AUSSCHEIDUNG SCHNEE- ODER NEBELFÖRMIGER SUBSTANZEN AUS LANGSAM STRÖMENDEN GASEN

A. WEHRLI UND E. KOVÁTS

*Organisch-chemisches Laboratorium der Eidgenössischen Technischen Hochschule, Zürich (Schweiz)*

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Die Ausscheidung organischer Dämpfe aus langsamen Gas-Strömen bereitet in einigen Fällen Schwierigkeiten. Organische Substanzen—besonders von höherem Molekulargewicht (Siedepunkt)—neigen beim Ausfrieren oft zur Nebelbildung. Die entstehenden festen oder flüssigen Schwebestoffe entziehen sich dann meist infolge ihrer durch Oberflächenenergie oder elektrische Aufladung bedingten Stabilität, der Wirkung des rekuperativen Systems, sodass in ungünstigen Fällen fast die gesamte Substanzmenge verloren geht.

Mit der Verbreitung der präparativen Gas-Chromatographie war man genötigt, manuell oder mit Hilfe einer Automatik bedienbare Kühlsysteme für dieses Problem zu entwickeln. Dies geschah durch:

(a) Wahl einer geeigneten Kühltaschenform[1–5],

(b) Verwendung eines Füllmaterials,

(c) Ausscheiden der Teilchen an Elektroden.

Diese Kühlsysteme verbessern somit die Ausbeute entweder durch Steigerung der Stosswahrscheinlichkeit der Teilchen mit der Wand—durch Erhöhung des Verhältnisses Wandoberfläche: Kühltascheninhalt (a und b)—oder durch elektrische Anziehungskräfte.

Die Stosswahrscheinlichkeit wird stark erhöht, wenn die Kühltasche mit einem Füllmaterial wie Glaswolle[6,7], Glaspulver[6], oder Metallwolle gefüllt ist. Die Kühltasche kann auch mit einem Adsorbenten, z.B. mit aktivem Aluminiumoxyd[3,8] oder Aktivkohle[6,8–10] beschickt werden. Für ganz kleine Substanzmengen wurde mit Erfolg ein einfaches, gekühltes U-Kapillarrohr verwendet[11,12]. Eine wesentliche Verbesserung der Ausbeute wurde auch mit Hilfe von Cotrell-Abscheidern[13] erreicht.

Fast alle diese Kühlsysteme haben die Nachteile, dass entweder die kleine Substanzmenge auf einer grossen Kühlfläche verteilt ist, sodass ein nachträgliches Sammeln der Substanz bedeutende Schwierigkeiten bereitet, oder dass bei engen sowie bei gefüllten Kühlfallen gerne Verstopfung eintritt.

In dieser Arbeit möchten wir ein Kühlsystem beschreiben, welches mit Erfolg für präparative gas-chromatographische Arbeiten angewendet wurde.

Das Gasgemisch wird in einem dickwandigen, mit einer dünnen Glasschicht isolierten Silberrohr aus dem Gas-Chromatographen ausgeführt. Dadurch wird eine frühzeitige Kondensation vermieden. Dieses Rohr ragt unmittelbar in eine rotierende Kühltasche. Das starke Temperaturgefälle auf der kurzen Distanz zwischen dem heissen Ende des Austrittsrohrs und der Kühltaschenwand begünstigt die Bildung von Nebel, welcher durch die Zentrifugalkraft an die Kühltaschenwand geschleudert wird. Durch verschiedene Glaseinsätze in das rotierende Kühlsystem können verschiedene Mengen (0.1–5 g) flüssiger oder fester Substanz gehandhabt werden. Das System hat den Vorteil, dass die Substanz ohne Verstopfungsgefahr in einer sehr kleinen Kühltasche in relativ hoher Ausbeute (*ca.* 90 %) zurückgewonnen, ferner, dass es auf einfache Weise ohne Verwendung von Hahnen oder Ventilen automatisiert werden kann.

## BESCHREIBUNG DER APPARATUR

Das Kühlsystem besteht aus drei Teilen: dem Kühlgefäss (K) mit zwei Zentrifugen (Z1) und (Z2), dem Fahrgestell (F) mit Hebel (H) und Elektromotor (E) und dem Gestell (G). Das Kühlgefäss (K) ist um die vertikale Achse drehbar, sodass durch eine

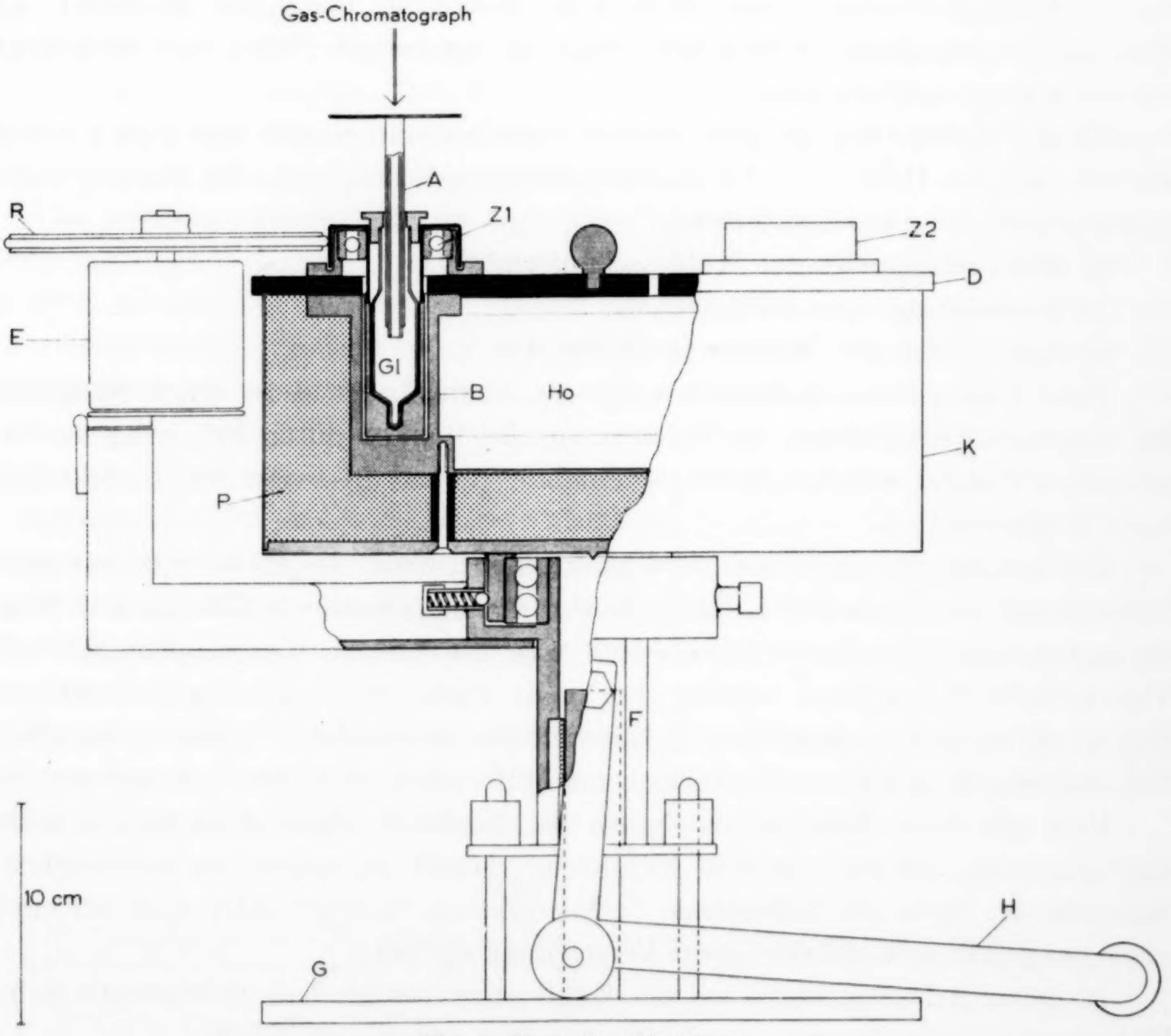

Fig. 1. Zentrifugalkühler.

Drehung um 180° die zweite Zentrifuge (Z2) durch das Friktionsrad (R) des Elektromotors (E) angetrieben werden kann. Die Zentrifugen (Z1) und (Z2) drehen sich in den Ausfräsungen des Aluminiumblockes (B). Durch Füllen des Hohlraumes (Ho) mit Kühlmittel kann dieser Block gekühlt werden. Der Block (B) ist mit Polystyrolschaum (P) isoliert und mit einem PVC-Deckel (D) gedeckt. In den Glaseinsatz (Gl) des drehenden Kühlfingers der Kühlzentrifuge (Z1) ragt das isolierte silberne Austrittsrohr (A) des Gas-Chromatographen, wenn das Fahrgestell (F) durch Hinunterdrücken des Hebels (H) gehoben wird, wie in Fig. 1 gezeigt ist. Das Fahrgestell ist an einer Achse mit Hilfe einer Kugelbüchse gelagert, diese Montage erlaubt ein präzises Senken und Heben des Fahrgestells, sowie des damit verbundenen Elektromotors und Kühlgefässes. Der Fraktionswechsel wird durchgeführt indem:

(a) durch Heben des Hebels (H) die Zentrifuge (Z1) gesenkt wird,

(b) durch Drehen des Kühlgefässes um 180° die zweite Zentrifuge (Z2) in Position gebracht und hier durch den Elektromotor (E) angetrieben wird, und

(c) durch Drücken des Hebels (H) die zweite Zentrifuge (Z2) zur Fraktionsentnahme gehoben wird.

Die zum Wechsel nötige Zeit beträgt etwa zwei Sekunden. Während mit der zweiten Zentrifuge eine Fraktion genommen wird, kann der Glaseinsatz der ersten Zentrifuge bequem gewechselt und somit die weitere Fraktionsentnahme vorbereitet werden. Die Drehzahl der Zentrifuge beträgt etwa 7000 U/min.

## DISKUSSION

Der Wirkungsgrad der Zentrifuge wurde am Beispiel des Citronellols untersucht, da die Rückgewinnung dieser Substanz wegen starker Nebelbildung Schwierigkeiten bereitete. Dabei zeigte sich, dass mit steigender Drehzahl der Verlust geringer wurde. Selbst bei kleinen chromatographierten Mengen (30 mg Citronellol in einer präparativen Kolonne von 1.5 cm lichter Weite und 250 cm Länge, bei 210°), hohen Strömungsgeschwindigkeiten und Kühlen der Kühltaschen auf — 10°, vermochte eine Drehzahl von 5000 bis 9000 U/min den Verlust auf etwa 10 % zu verringern. Eine ähnliche Ausbeute wurde durch Hintereinanderschalten von drei auf — 80° gekühlten, nicht rotierenden grossen Kühltaschen (Inhalt 200 ml; ungefüllt) erreicht.

Die Kühlzentrifuge wurde zum Ausfrieren zahlreicher Monoterpen-Kohlenwasserstoffe, Ketone und Alkohole verwendet. Da sie sich gut für kleine Mengen eignet, bewährte sie sich auch für das Sammeln von Nebenkomponenten. Bei diesen wurde die geringe Substanzmenge (0.5 mg) durch nachträgliches Zentrifugieren in die verjüngte Spitze der Kühltasche getrieben*. Substanzmengen dieser Grössenordnung waren mit Hilfe grosser, ungefüllter Kühltaschen nicht fassbar.

Für die Rückgewinnung von Substanzen mit niedrigem Siedepunkt zeigte sich das Kühlsystem in praxi ungeeignet, weil das Kühlen der Kühltaschen auf tiefere Temperaturen als — 30° Schwierigkeiten bereitete; in diesem Falle gaben einfache tiefgekühlte Kühltaschen bessere Ausbeuten. Wir möchten ferner nicht behaupten, dass bei hochsiedenden Substanzen durch Verwendung von gefüllten oder ungefüllten Kühltaschen genügenden Inhaltes nicht bessere oder sogar quantitative Ausbeuten erreicht werden können, jedoch verliert man dadurch die eingangs erwähnte Handlichkeit der rotierenden Kühltasche.

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* Das untere Ende der Kühltasche wurde verjüngt (siehe Fig. 1), sodass beim Zentrifugieren die kleine Substanzmenge sich in einem engen Rohr sammelt.

## DANK

Die Autoren danken der Firma Firmenich & Co. in Genf für die Unterstützung dieser Arbeit, insbesondere auch Herrn Dr. E. PALLUY für die Lösung und Ausführung von Konstruktions-Details.

Die Fertigmontage wurde von der Firma H. Lüdi & Cie., Zürich, ausgeführt.

## ZUSAMMENFASSUNG

In dieser Arbeit wurde ein Zentrifugalkühler für präparative gas-chromatographische Arbeiten beschrieben. Er gestattet organische Substanzen aus dem Eluenten mit Hilfe einer kleinen rotierenden Kühltasche in relativ hoher Ausbeute zurückzugewinnen.

## SUMMARY

A rotating cooling trap has been designed for preparative gas-chromatographic work. This permits recovery of an organic substance from the eluent in a small container with relatively low loss.

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# RELATION BETWEEN THE STRUCTURE AND THE CHROMATOGRAPHIC BEHAVIOUR OF FURAN, PYRROLE AND THIOPHENE DERIVATIVES

JAROSLAV FRANC

*Research Institute for Organic Syntheses, Pardubice-Rybitví (Czechoslovakia)*

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A comparison of the number of papers concerned with the paper chromatography of aromatic and heterocyclic compounds, shows that those dealing with heterocyclic compounds are decidedly in the minority. The same fact applies to studies of the chromatographic behaviour of these compounds in relation to their structure. The situation is, in general, rendered more difficult by the circumstance that no profound conclusions can be drawn from a study of the chromatographic behaviour of arbitrarily chosen compounds, the structures of which are only similar to a limited extent.

Recently we had the opportunity of studying the chromatographic behaviour of several mono- and dicarboxylic acids derived from furan, pyrrole and thiophene and in the present paper we have attempted to explain some of the phenomena and relations observed.

## EXPERIMENTAL PART

The chromatographic separation of the acids given in Table I was effected by the descending technique on Schleicher & Schüll paper 2045b (very slow). The acids were applied on the starting point as pyridine solution, quantities of about 25 µg being

TABLE I

$R_F$ VALUES OF MONO- AND DICARBOXYLIC ACIDS

Solvent system: *n*-butanol–ethanol–pyridine–water (3:1:1:1). Paper: Schleicher & Schüll 2045b. Temp.: 20° ± 0.5°.

| *Acid* | $R_F$ | $(R_F)_{BA} - R'_F$* | $(R_F)_{MA} - (R_F)_{DA}$* |
|---|---|---|---|
| Benzoic | 0.59 | | |
| Terephthalic | 0.215 | | $0.37_5$ |
| Furan-2-carboxylic | 0.30 | 0.21 | |
| Furan-2,5-dicarboxylic | 0.063 | | 0.42** |
| Thiophene-2-carboxylic | 0.46 | 0.05 | |
| Thiophene-2,5-dicarboxylic | 0.10 | | 0.44** |
| Pyrrole-2-carboxylic | 0.41 | 0.10 | |
| 2,5-Dimethylpyrrole-3,4-dicarboxylic | 0.17 | | 0.40*** |

* BA = benzoic acid; MA = monocarboxylic acid; DA = dicarboxylic acid.

** For all differences between the $R_F$ values, which lie outside an $R_F$ range of 0.16–0.84, the calculation of the $R_M$ function was used.

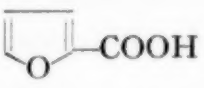
*** =CH– increments (+ 0.08) added.

used. The chromatograms were developed with a butanol–ethanol–pyridine–water mixture (3:1:1:1) for a period of 16–17 hours. Detection was carried out by spraying the chromatograms with a saturated aqueous solution of 2,6-dichlorophenol-indophenol; the acids give pink spots on a blue background.

In order to prevent variations in the $R_F$ values, the chromatographic procedure was performed in a room where the temperature was kept constant. The $R_F$ values given in Table I are averages of the values obtained from 10 selected chromatograms.

## RESULTS AND DISCUSSION

As can be seen in Table I two aromatic acids—benzoic acid and terephthalic acid—were also chromatographed besides the heterocyclic acids. This was done in order to study the differences between the heterocyclic and aromatic nucleus. First, the monocarboxylic acids were compared. The $R_F$ values of these acids increased in the following order: furan-2-carboxylic acid (I), pyrrole-2-carboxylic acid (II), thiophene-2-carboxylic acid (III), benzoic acid.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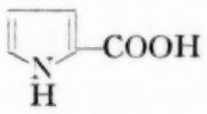
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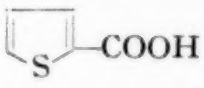

III

All the above-mentioned heterocyclic acids have one =CH– group less than benzoic acid. Previously we had computed[1] that each additional =CH– group causes an increase in the $R_F$ value of about 0.08 unit in the solvent system used. Therefore, for an adequate comparison this increment should be added to the $R_F$ values of all the heterocyclic acids. In this way a modified value $R'_F$, is obtained:

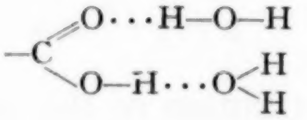
| | $R'_F$ |
|---|---|
| Thiophene-2-carboxylic acid | 0.54 |
| Pyrrole-2-carboxylic acid | 0.49 |
| Furan-2-carboxylic acid | 0.38 |

On comparing these calculated values with the $R_F$ value of benzoic acid (0.59), it is evident that thiophene-2-carboxylic acid exhibits the most pronounced "aromatic" character. This observation is in full agreement with the data of other authors[2,3], who have studied the electron structure of thiophene derivatives. The $R_F$ values of the other acids decrease in a certain order. This decrease in the $R_F$ value can be explained in the same way as the chromatographic separation of similar derivatives, which differ in that they form intermolecular hydrogen bonds of various energy content with the stationary phase. It is known that bonds of the type O–H···O have the highest energy levels; those of the O–H···N type come next and those of the O–H···S type have the lowest energy. On comparing the differences $(R_F)_{\text{benzoic acid}} - R'_F$ (given in Table I), it is evident that they can be arranged in the same order as the energy levels of the hydrogen bonds of these different types. If the previously calculated chromatographic equivalent of the hydrogen bond $R_E = 1.3 \pm 0.1$ kcal/mol (this value corresponds to the change in $R_M$ that occurs if the chelate-bond energy is varied by 1.3 ± 0.1 kcal/mol)[4] is used in the calculation of these energy levels, the following values of the bond energy are obtained:

| | |
|---|---|
| O–H···O | 5.5 ± 0.5 kcal/mol |
| O···H–N | 2.6 ± 0.3 kcal/mol |
| O–H···S | 1.3 ± 0.1 kcal/mol |

These values are in good agreement with the data obtained by another method[5].

Next, the behaviour of the dicarboxylic acids was studied. The introduction of another –COOH group into the molecule gives rise to a considerable decrease in the $R_F$ value. Calculation of the differences between the $R_F$ values of mono- and dicarboxylic acids (see Table I) shows that these differences are practically constant. The average difference (0.41) is in very good agreement with prior observations for another group of aromatic acids[1]. If we examine this value more closely, we find that it is nearly twice as great as that computed for the difference between the $R'_F$ value of furan-2-carboxylic acid and the $R_F$ of benzoic acid, *i.e.* it corresponds to two hydrogen bonds of the O–H···O type:



It is possible to make a further comparison, namely between dicarboxylic acids. For purposes of calculation the increment for one =CH– group (+ 0.08) is again added to the $R_F$ values; thus the following values are obtained:

| | $R'_F$ |
|---|---|
| Furan-2,5-dicarboxylic acid | 0.09 |
| 2,5-Dimethylpyrrole-3,4-dicarboxylic acid | 0.12 |
| Thiophene-2,5-dicarboxylic acid | 0.14 |

The same calculation as in the case of the monocarboxylic acids (*i.e.* comparing these data with the $R_F$ value of terephthalic acid) leads to the following results:

| | | |
|---|---|---|
| O–H···O | $\Delta R_F$ 0.23 | 5.95 ± 0.6 kcal/mol |
| O···H–N | $\Delta R_F$ 0.14 | 3.6 ± 0.4 kcal/mol |
| O–H···S | $\Delta R_F$ 0.11 | 2.8 ± 0.3 kcal/mol |

Whereas the energy of an intermolecular hydrogen bond O–H···O of furan-2,5-dicarboxylic acid is, on the whole, the same as that of the monocarboxylic acid, this value is somewhat increased in the case of pyrroledicarboxylic acid. In the case of thiophene-2,5-dicarboxylic acid, however, a substantial increase is observed (to approximately twice the value). The only explanation for this phenomenon would seem to be the same that KESWANI AND FREISER[6] suggested for the fact that the observed dipole moments of thiophenealdehyde and acetylthiophene were greater than the values calculated by the vector method. According to these authors the difference is caused by the resonance effect. In this case, the negative electrical charge at the sulphur atom increases as well, so that the energy of the intermolecular hydrogen bond rises.

Further, we shall discuss the possibility of making a rough estimation of the bond angles of carboxylic groups. In one of our preceding papers we deduced a relation between the chromatographic behaviour and the dipole moments of aromatic isomers[7]:

$$R_F = K - 0.1\,\mu \quad \text{for an} \quad R_F \text{ range of } 0.16 - 0.84$$

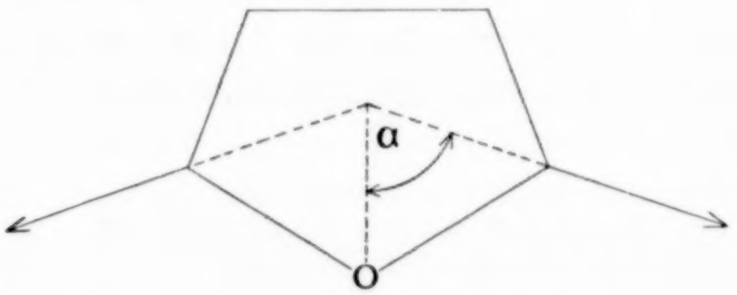
and

$$R_M = C + 2.3 \log \frac{1 + 0.1\,\mu}{1 - 0.1\,\mu}$$

If we assume that the two carboxyl groups of terephthalic acid are so arranged that they form an angle of 180°, then if the angle between the carboxyl groups of furan-2,5-dicarboxylic acid were also 180°, the difference between the dipole moments of furan-2-carboxylic acid and furan-2,5-dicarboxylic acid would be the same as that between benzoic acid and terephthalic acid. In the case of furan-2,5-dicarboxylic acid, however, this angle must evidently have another value and, consequently, the difference between the dipole moments cannot be the same. The dipole moment of furan-2,5-dicarboxylic acid can be calculated as the vector sum of the dipole moments of the functional groups, with the formula

$$\mu = 2 \cos \alpha \cdot \mu'$$

in which $\mu'$ designates the contribution of the group dipole moments to the total dipole moment of the molecule.



By calculating $\mu$ from the equation $R_F = K - 0.1\,\mu$ and substituting for $\mu'$ the value 1.7, we find that $2\alpha = 144°$. If we assume that the $R_F$ value is determined with an accuracy of $\pm$ 0.01, then the error in the estimation of the angle will be $\pm$ 7°.

If we assume that the carboxyl group divides the corresponding –O–C=C– angle into two equal parts and use the data obtained by SCHOMAKER AND PAULING[8], for the calculation of the furan angle, the same value for $2\alpha$ is obtained. A similar calculation can also be made for thiophene-2,5-dicarboxylic acid. The dipole moment calculated in this case is lower than the one found; this fact can also be explained by the above-mentioned resonance effect.

With this example we have attempted to show what relations may be revealed by comparing a few $R_F$ values if suitable series of similar compounds are chosen. However, it need not be pointed out that the accuracy in determining the $R_F$ values should be as high as possible.

## ACKNOWLEDGEMENT

I wish to thank Mr. JANDA (Department of Organic Chemistry, University of Chemical Technology, Prague) for kindly providing the pure acids.

## SUMMARY

The chromatographic behaviour of mono- and dicarboxylic acids derived from benzene, furan, pyrrole, and thiophene has been studied. The energy levels of intermolecular hydrogen bonds of heterocyclic acids have been estimated and an attempt has been made to calculate the bond angles of furan-2,5-dicarboxylic acid.

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# LIQUID–LIQUID PARTITION CHROMATOGRAPHY

## SEPARATION OF THE 2,4-DINITROPHENYLHYDRAZONES OF SATURATED ALDEHYDES, METHYL KETONES, 2-ENALS AND 2,4-DIENALS

EDGAR A. CORBIN, DANIEL P. SCHWARTZ AND MARK KEENEY
*Dairy Products Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Washington, D. C. (U.S.A.)*

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Investigators attempting to characterize the normal and abnormal flavors in food products are directing more and more attention to the carbonyl compounds associated with such flavors. After a preliminary isolation, these carbonyl compounds are usually converted into 2,4-dinitrophenylhydrazone derivatives. Separation, identification and quantitative estimation of these colored derivatives are then made. Separation of the 2,4-dinitrophenylhydrazones is accomplished by chromatography, either on paper or on a partition column. There is a definite need for a chromatographic method which can separate a variety of 2,4-dinitrophenylhydrazones in a rapid, consistent, and quantitative manner. The partition column method was selected for study since it is more adaptable to quantitative measurement.

The partition chromatographic method of KRAMER AND VAN DUIN[1] as modified by MONTY[2] and BASSETTE[3] uses nitromethane as a stationary phase, supported on celite, and purified hexane as the mobile phase. This method, fine as it is, was found to have two main disadvantages. The first involves the use of nitromethane. Since nitromethane absorbs light strongly in the same region as the 2,4-dinitrophenylhydrazones a quantitative spectrophotometric measurement of the color in the eluate fractions can be made only after evaporating away the nitromethane and dissolving the residue in another solvent. The second disadvantage is that the detection and fractionation of the colored derivatives is done visually which may allow some small amounts of derivatives to pass by unseen.

In the present method these two disadvantages, we feel, have been satisfactorily alleviated. To replace the nitromethane, acetonitrile or 2-chloroethanol was found to be just as efficient and since neither absorbs light in the same region as the 2,4-dinitrophenylhydrazones, the eluate is ready for spectrophotometric measurement without any alteration. To detect the chromatographic bands, especially those invisible to the eye, the eluate is collected in 10 ml fractions and an absorbance measurement is made of each fraction as it is collected. Very small quantities of 2,4-dinitrophenylhydrazones can be detected in this way and a quantitative measure of the amount present is obtained at the same time.

The partition chromatographic method to be described is equally successful in separating the 2,4-dinitrophenylhydrazones of saturated aldehydes, saturated methyl ketones, 2-enals, or 2,4-dienals.

## EXPERIMENTAL

### *Apparatus and reagents*

*Beckman Model B spectrophotometer* with test tube holder and a minimum of three optically matched test tubes (15 × 95 mm or larger).

*Measuring siphon* to deliver 10 ml ± 0.2 ml.

*Chromatographic tube* approximately 25 × 350 mm with constricted tip and removable solvent reservoir. A permanent sintered-glass plate or pyrex wool plug can be used at the bottom of the tube.

*Tamping rod.* A stainless steel disc (1 × 20 mm) is perforated by drilling 20 to 24 $^1/_{16}$ in. holes in it. This disk is soldered onto the end of a stainless steel rod (5 × 450 mm) to form a T-shaped tamping rod.

*Purified hexane.* Reflux 3 l of high-purity *n*-hexane (Phillips Petroleum Company) with 50 ml concentrated sulfuric acid for 3 hours. Decant hexane from acid and wash with two 500 ml portions of distilled water. Decant hexane into distilling flask, add 3 teaspoons of sodium hydroxide pellets and mix. Distil, collecting the 69–70° fraction.

*Acetonitrile.* Distil and collect the 80–81.5° fraction.

*2-Chloroethanol.* Distil and collect the 127–129° fraction.

*Dry celite.* A sufficient quantity of analytical grade celite* is dried in a 160° oven for at least 24 hours before use.

*Equilibrated hexane for chromatography.* Purified hexane is shaken with an excess of acetonitrile or 2-chloroethanol and the hexane layer used.

### *Column preparation*

*Acetonitrile column.* Twenty five grams of dried celite are placed in a blendor jar with 250 ml of hexane equilibrated with acetonitrile and blended for one or two minutes. Five-tenths ml of distilled water and 33 ml of acetonitrile are added slowly to the swirling suspension. When completely homogenous the celite slurry is poured through a funnel into the chromatographic tube, the tip of which has been closed with a piece of tubing and pinchcock. The tamping rod is moved through the slurry to remove all air bubbles. With the pinchcock opened, air pressure (6–8 lb./in.$^2$) is applied to the top of the column until the celite is compact, being careful that the celite is always covered with equilibrated hexane. More slurry is added, stirred, and compressed as before, until all the celite has been added. The tamping rod is used to firm and level the top of the column material. Flow-rate at this point should be 120–150 drops/min. A prepared column can be kept a week or more if necessary by closing the pinchcock at the bottom and stoppering the top to prevent evaporation of the hexane covering the column material.

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* Johns-Manville analytical filter aid.

*2-Chloroethanol column*. This column is prepared in exactly the same manner as the acetonitrile column except that purified hexane saturated with 2-chloroethanol is used and 25 ml of 2-chloroethanol replace the 33 ml of acetonitrile. The flow-rate of a chloroethanol column is less than that of an acetonitrile column. By using slightly less air pressure to compress the column material a satisfactory flow-rate will result.

### *Column operation*

Both columns are set up and operated in the same manner. The following method of operation has been found to give fast and reproducible results. The prepared column is supported near the spectrophotometer so both may be attended simultaneously. The 2,4-dinitrophenylhydrazones should be dissolved in 10 ml or less of equilibrated hexane. If necessary purified hexane can be substituted. When the volume of solvent covering the column is one ml or less the 2,4-dinitrophenylhydrazone solution is carefully added. Collection of the eluate is started immediately. When the sample solution has drained into the column, 2 ml portions of equilibrated hexane are added until no color can be seen in the hexane. The solvent reservoir is attached and filled with equilibrated hexane. The siphon is situated to receive the column eluate and to empty each 10 ml fraction directly into one of the matched spectrophotometer test tubes. The optical density of each 10 ml fraction is then measured and recorded while the next fraction is being collected. Equilibrated hexane is used as the reference blank. Since the optical density measurement is less time-consuming than the collection of the next fraction, a method of increasing the flow-rate of the column, when needed, was introduced. An air line adjusted to 1 or 1 ½ lb./in.$^2$ pressure is connected to the solvent reservoir. At some convenient point in this air line a "T" tubing connection is inserted. When the open end of the "T" tube is closed, by placing a finger over it, air pressure is directed against the solvent in the reservoir increasing the flow-rate of the column. Pressure is released just as the fraction starts to siphon. About fifty 10 ml fractions can be collected and measured in one hour using this procedure.

## DISCUSSION

Figs. 1 and 2 show the separations that can be expected from the two columns when they separate the 2,4-dinitrophenylhydrazones of the four homologous series studied. The subscripts ($C_3$, $C_{10}$, etc.) indicate the number of carbon atoms in the parent aldehyde or ketone. No attempt was made to chromatograph equal molar amounts of each derivative. To conserve space the formaldehyde ($C_1$) peak is not shown in Figs. 1(A) and 2(A). The formaldehyde curve would reach a maximum within fractions 210–215 in Fig. 1(A) and within fractions 255–260 in Fig. 2(A). The wavelength at which each fraction of a given series is measured in the spectrophotometer is also shown. These wavelengths of maximum absorption were determined on a mixture of appropriate 2,4-dinitrophenylhydrazones. Individual 2,4-dinitrophenylhydrazones may give maxima which differ slightly from these average values. When dealing with unknown compounds the wavelength at which the readings should be made will also be unknown. In this case initial readings are made at 335 m$\mu$; then one fraction from each "peak" is set aside to be measured at the other wavelengths (340, 355, and 370 m$\mu$). The wavelength which gives the greatest absorbance places the unknown tentatively in one of the four classes, aldehyde, methyl ketone, enal, or dienal.

"Peak volume" is defined as that volume of mobile phase required to elute the fraction of deepest color for each 2,4-dinitrophenylhydrazone. Peak volume is unaffected by the amount of material being chromatographed. Corrected peak volume is the peak volume minus the hold up volume, hold up volume being the amount of mobile phase held on the column. The hold up volume for a 25 g column (acetonitrile or chloroethanol) is 60 ml. This 60 ml value was obtained by weighing the chromatographic tube, the celite, and stationary phase needed to make a column and subtracting these weights from the total weight of the completed column. The completed column was weighed with no excess solvent present. The difference in weights was

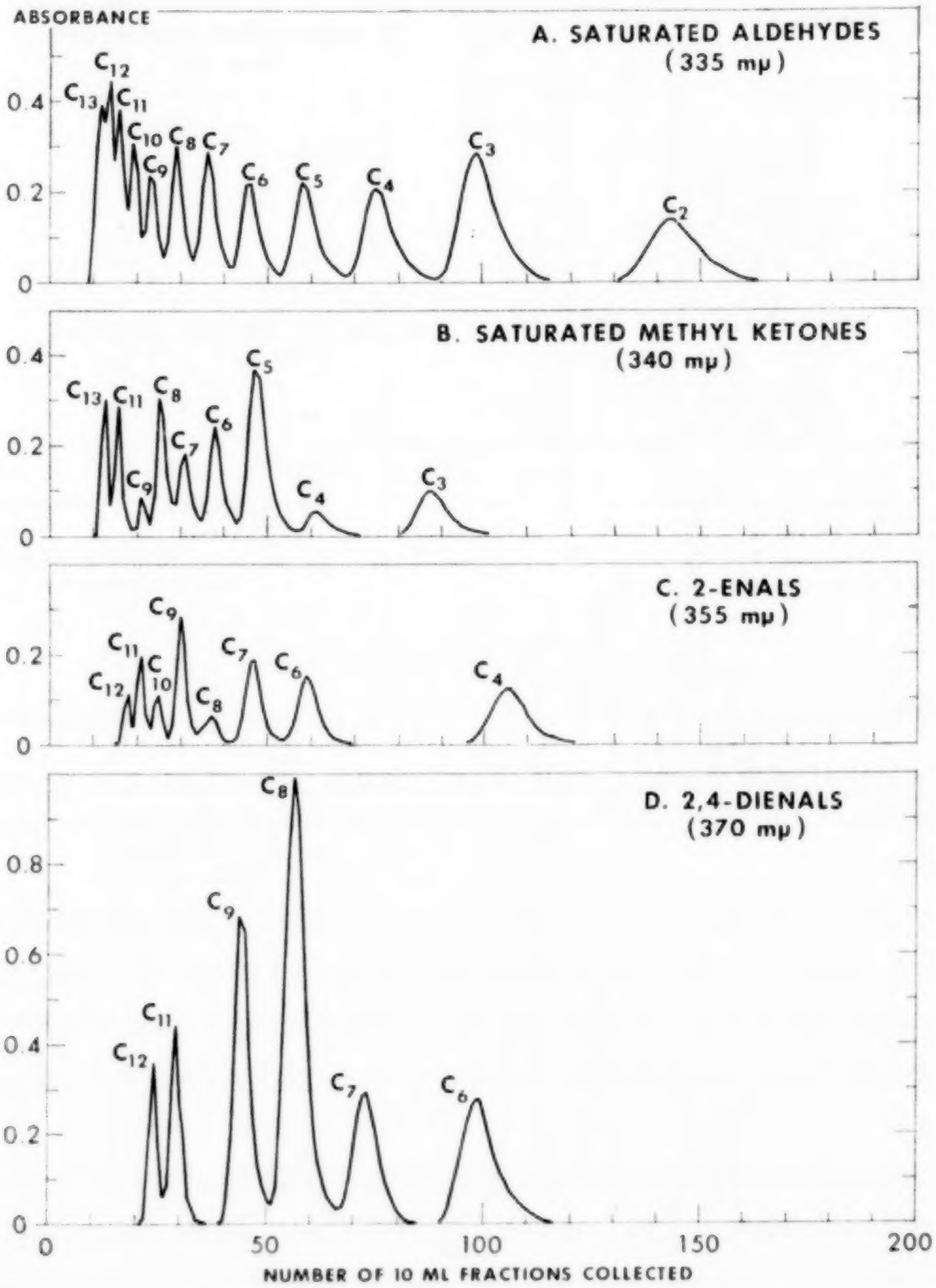

Fig. 1. Typical chromatograms using the acetonitrile column.

converted into ml of mobile phase. This corrected peak volume is very similar to, but not identical with, the true retention volume described by KRAMER AND VAN DUIN[1]. These authors plotted the logarithms of their true retention volumes against the number of carbon atoms in the parent compound of two homologous series and obtained practically straight lines. A truly straight line would be taken to indicate an ideal liquid–liquid partition chromatographic system. Similar plots of our data, using corrected peak volumes, are shown in Figs. 3 and 4. All the lines are curved indicating non-ideality in the present partition systems. This deviation from ideal is attributed to the high moisture content of the columns, since some columns prepared without water gave plots which were not so curved.

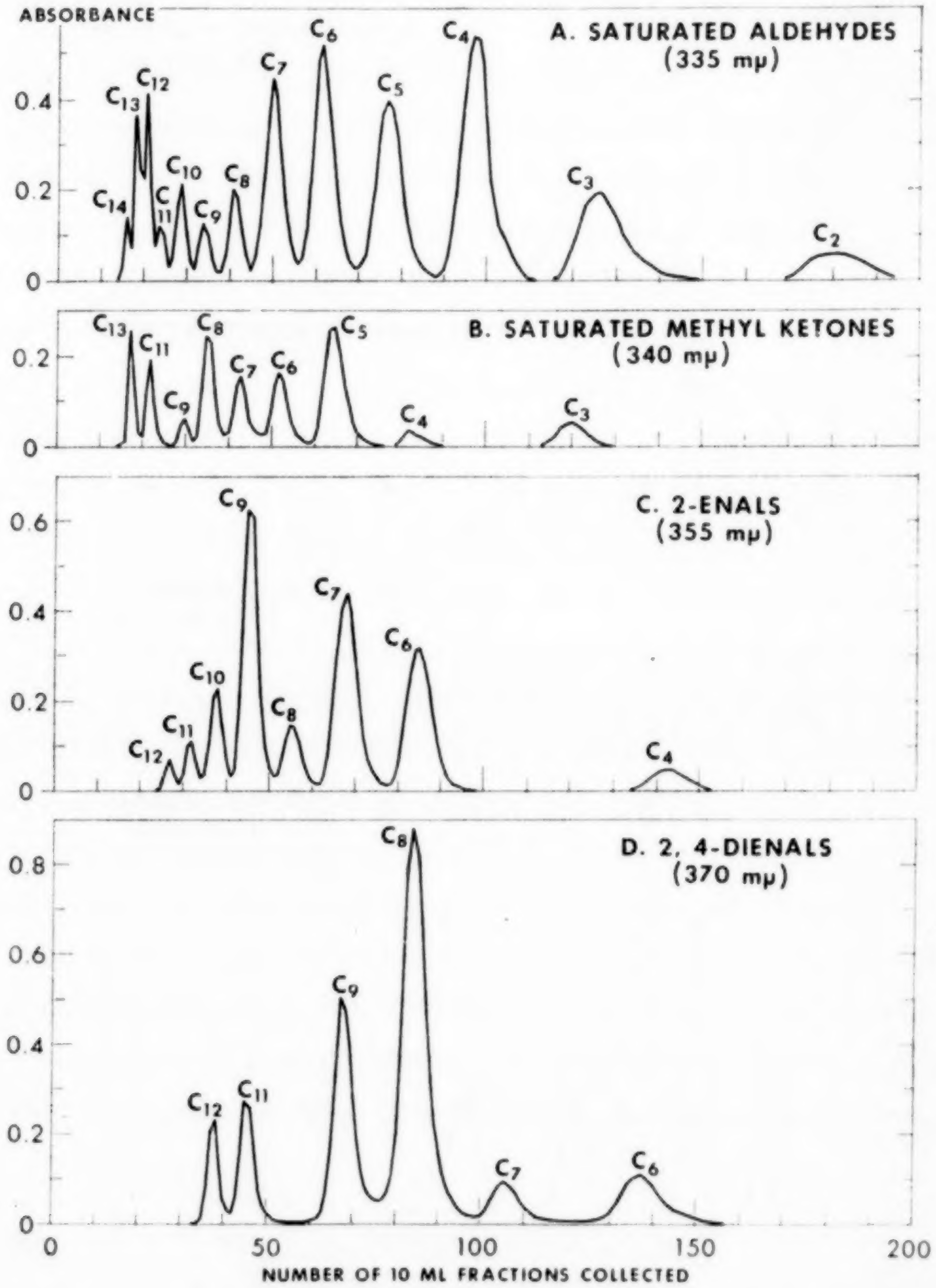

Fig. 2. Typical chromatograms using the 2-chloroethanol column.

A high moisture content is desirable in the acetonitrile column and a necessity in the chloroethanol column. In both columns the moisture present improves the resolving power, prevents cracking, channeling, or distortion of the top surface during preparation and manipulation of the column, and decreases the eluate flow to a more desirable rate. The eluate from a chloroethanol column prepared without water is cloudy and impossible to measure in the spectrophotometer. The water (0.5 ml) added during column preparation usually alleviates this cloudiness. However, if a cloudy eluate is noticed, or suspected, a drop of absolute ethanol mixed into each fraction will quickly remove any cloudiness present.

Recovery data were obtained by measuring the optical density of a 2,4-dinitrophenylhydrazone solution, chromatographing 10 ml of this solution on a column, and measuring all color removable from the column. This was done with individual 2,4-dinitrophenylhydrazones and with mixtures. The majority of recoveries ranged from 92 % to 102 %. In general, low recoveries (92–95 %) were obtained from the slower moving bands and higher recoveries (95–100 %) from the faster moving bands. The formaldehyde, acetaldehyde, and acetone derivatives gave the lowest recoveries (85–90 %).

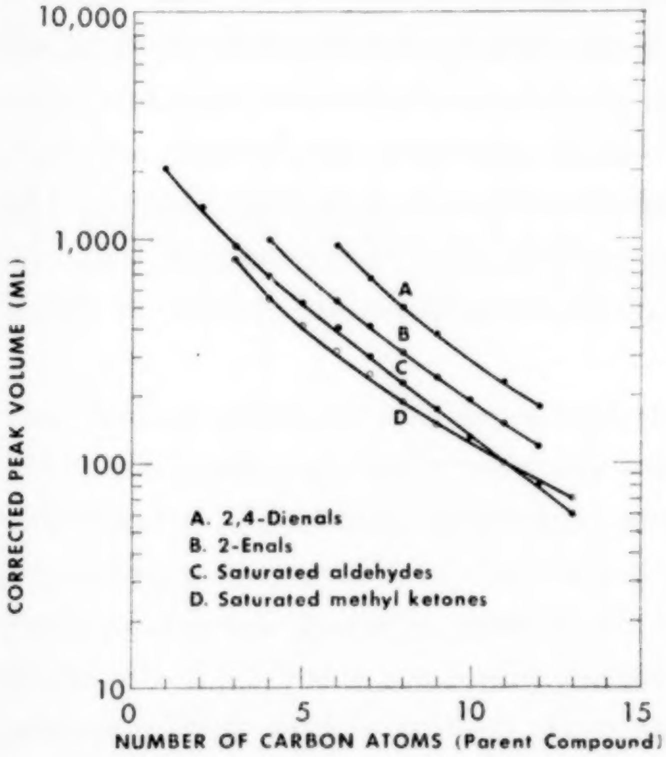

Fig. 3. Relationship of carbonyl chain length to peak volume, acetonitrile column.

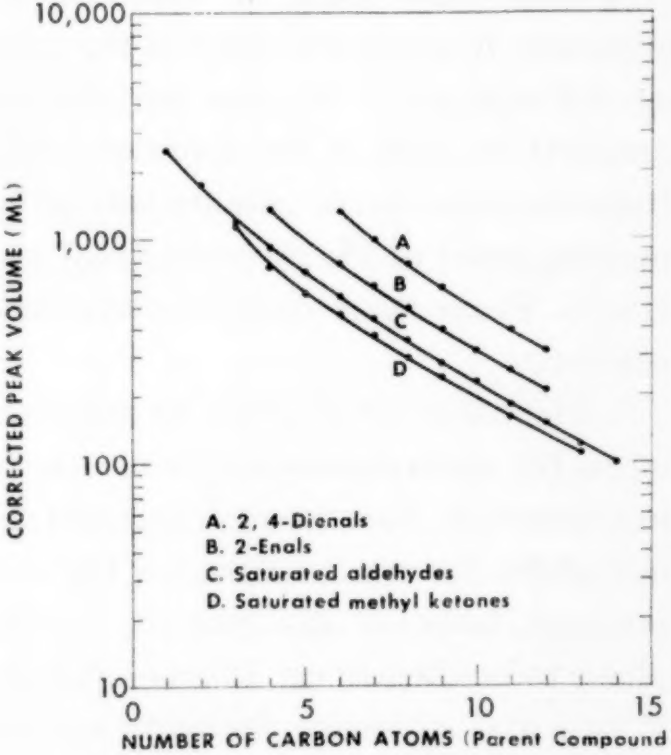

Fig. 4. Relationship of carbonyl chain length to peak volume, 2-chloroethanol column.

The method of detecting the 2,4-dinitrophenylhydrazone bands as they move off the column is inherently sensitive. The equilibrated hexane used as the reference blank and the clear column eluate should have the same optical density; any increase in eluate optical density is taken to indicate the presence of 2,4-dinitrophenylhydrazone. The method can be defined as being sensitive to 1 μg of 2,4-dinitrophenylhydrazone per 10 ml fraction since this amount will increase the optical density 0.005 to 0.010 units. Adjacent homologs, up to the $C_{10}$ derivative, can be qualitatively separated from each other when their concentrations differ by as much as 10 to 1. Adjacent $C_{11}$ to $C_{14}$ derivatives separate only when their relative concentrations approach equality. The columns are capable of handling milligram quantities of the 2,4-dinitrophenylhydrazones but best column performance is obtained by chromatographing 20 to 50 μg of the higher molecular weight derivatives ($C_{14}$ to $C_9$) and from 50 to 100 μg of the lower molecular weight compounds ($C_8$ to $C_1$).

Some investigators have reported the decomposition of 2,4-dinitrophenylhydrazones on some chromatographic columns. FORSS AND DUNSTONE[4] found that appreciable decomposition of a number of 2,4-dinitrophenylhydrazones took place on silica gel columns when chromatographed for 24 hours or longer, and that the acetone derivative was affected on an alumina column. BASSETTE[3] has reported complete destruction of the methional (3-methylthiopropanal) derivative on a silica gel–nitromethane column. No decomposition of any compound has been noticed or suspected while using the acetonitrile or chloroethanol columns but it was deemed advisable to study the effect of the columns on methional 2,4-dinitrophenylhydrazone since it appears to be of an unstable nature. The methional derivative was chromatographed on each of the columns; visually the bands appeared unaffected; recovery from the acetonitrile column was 92% and from the chloroethanol column 93% The melting point of the recovered material was the same as that of the original material (123°). From these data it was concluded that no decomposition occurs on either column.

The use of air pressure to increase the eluate flow is needed to conserve time only when the spectrophotometer measurement takes less time than the collection of the next fraction. This intermittent and moderate use of pressure against the column does not affect the peak volume or the symmetry of the curves. Excessive or prolonged pressure, however, disrupts the equilibrium of the column causing some stationary phase to be washed off. If small globules of stationary phase are observed in the eluate it indicates excessive flow-rate and in this case peak volume values will be smaller and spectrophotometer readings will be erratic.

When dealing with unknown compounds or where quantitative data are desired each prepared column should be used for only one determination. Using a chloroethanol column more than once under any circumstance is not recommended. The acetonitrile column holds up well under repeated use but should be used more than once only when it is known to be clean of all previous compounds and when the identity of the 2,4-dinitrophenylhydrazones is known.

Of the two columns studied the acetonitrile column has been found to be the more reliable and the least liable to present difficulties of operation. It has slightly less resolving power than the chloroethanol column, and as a result it takes less time and less solvent to remove a particular compound. The chloroethanol column is the reverse, more resolving power but more time-consuming in operation.

Although the two columns are satisfactory for separating homologs of one series, it can be seen from Figs. 1 and 2 that the separation of a mixture of compounds from all four series, aldehydes, ketones, enals, and dienals, would probably not be possible.

## ACKNOWLEDGEMENTS

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## SUMMARY

Two similar partition chromatographic systems are described. Acetonitrile or 2-chloroethanol is used as the stationary phase on a celite column and hexane saturated with acetonitrile or 2-chloroethanol is the mobile phase. Both are suitable for the separation of the 2,4-dinitrophenylhydrazones of saturated aldehydes, saturated methyl ketones, 2-enals, and 2,4-dienals. Details of column preparation and operation are given. Graphs indicating the separations to be expected from each of the four homologous series are included.

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# STUDIES ON GEL FILTRATION

## SORPTION PROPERTIES OF THE BED MATERIAL SEPHADEX

BERTIL GELOTTE
*Research Laboratories, Pharmacia, Uppsala (Sweden)*

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Gel filtration, as described recently[1], is a method that makes possible the separation of substances with different molecular dimensions, and it has been applied with success in our biochemical laboratory. We have used the column technique for desalting protein solutions and for the separation of proteins and other colloids from low-molecular-weight substances[6]. The method has also been used for group separation of protein hydrolysates and biological extracts.

We have observed that, under certain conditions, some substances are adsorbed to the bed material, whereas others may exhibit a negative sorption, at least for some part of the applied quantity. We thought it would be of interest to make a closer investigation of the sorption properties of the bed material, especially the sorption of low-molecular-weight substances.

This paper describes gel filtration experiments with buffering substances, amino acids, purine and pyrimidine derivatives, vitamins, alkaloids and some simple aromatic substances.

### PRINCIPLES OF GEL FILTRATION

A theory for gel filtration and a more detailed description of the method and the bed material will be presented by FLODIN[2].

In a packed column one can distinguish two kinds of aqueous phases, one within the gel grains and one surrounding the grains. Let us denote the sum of the internal aqueous volumes of the grains as the inner volume of the column, $V_i$, the volume of the surrounding aqueous phase as the outer or void volume, $V_o$, and the partition coefficient for a substance between these two phases as $K_D$. When a substance is filtered through the column, the elution volume, $V_e$, is

$$V_e = V_o + K_D V_i$$

A substance submitted to gel filtration is preferentially characterized by its $K_D$ value, which is calculated from the expression above as

$$K_D = \frac{V_e - V_o}{V_i}$$

A complete exclusion from the inner phase is generally obtained for large molecules such as proteins, which are hindered from entering the interior of the grains by the polysaccharide network. A low-molecular-weight solute such as glycine can diffuse freely into and through the grains and has a $K_D$ value of about 1.

In reality, there are some exceptions to these rules. The calculation of $K_D$ by the above equation is only approximately true. In fact, part of the inner volume, $V_i$, is water of hydration which is firmly bound to the polysaccharide framework in the gel grains and is inaccessible to the solute molecules. For an accurate determination of $K_D$, the inner volume should be corrected for the water of hydration.

A large number of substances, inlcuding many of those discussed in this paper, interact with the bed material. Thus, some of them are slightly adsorbed and their elution is delayed, which results in an increased $K_D$ value; others show a negative sorption, and have a lower $K_D$ than expected.

## MATERIALS

The filtration experiments were made with columns packed with the cross-linked polysaccharide Sephadex G-25 (manufactured by Pharmacia, Uppsala, Sweden). The characteristics of the bed material were: water regain = 2.9 g water/g dry substance; wet density = 1.099. In the majority of experiments, the grain size in the dry state was 50–100 mesh, and in some experiments it was 200–400 mesh.

Though the bed material should be completely non-ionized, it was found to contain a small amount of ionized groups. These are probably carboxyl groups, and they amount to about 10 $\mu$equiv./g dry Sephadex.

The test substances used in the filtration experiments were of analytical grade or a comparable purity.

## METHODS

The dry Sephadex was allowed to swell in 0.05 $M$ sodium chloride for half an hour and was then freed from fine-grained material by repeated sedimentations and decantations. The gel grains were poured into a glass tube and packed in the same manner as is described for cellulose columns in zone electrophoresis[3]. A circular filter paper was put on the top of the packed column to protect the surface. Most of the experiments were made on the same column with the dimensions of 3.5 × 35 cm. After equilibration of the column with distilled water or with an electrolyte solution, the substances to be tested were put on the column in a volume of 5.0 ml. Elution was then started with the same aqueous solution as was used for equilibration with a hydrostatic pressure of 60 cm. This gave a flow rate of 2 ml per minute. When an effluent volume somewhat smaller than the void volume of the column had been taken off, the rest of the effluent was collected in fractions of 4.8 ml in an automatic fraction collector. All experiments were carried out at room temperature.

The fractions were analysed by a suitable method: acids and bases by titration with dilute sodium hydroxide and hydrochloric acid, respectively; salts by titration after passage of an anion or cation exchanger; amino acids, except tryptophan and tyrosine, with ninhydrin reagent according to MOORE AND STEIN[4]; tryptophan, tyrosine and other aromatic substances by measurement of their ultraviolet absorption in a Unicam SP 500 spectrophotometer.

The substances to be tested were separately filtered through the column and the yield and the $K_D$ value determined.

The data necessary for the calculation of $K_D$ are determined in the following way. The elution volume is determined by measuring the effluent volume from the addition of the test solution to the point where the concentration gradient of the eluted substance is maximum. $V_0$ is experimentally determined as the elution volume for haemoglobin, with phosphate buffer (ionic strength $\mu = 0.05$) pH 7.0 as eluant. $V_i$ is calculated from the water regain ($W_R$) and the dry weight of bed material ($a$).

$$V_i = a \cdot W_R$$

No correction for the water of hydration is made. A $K_D$ value of 0.8 therefore indicates a non-restricted diffusion in the gel column.

## RESULTS

The applicability of the gel filtration method has been shown by FLODIN[2], and experiments with proteins, peptides, and amino acids are described by PORATH[5,7]. The aim of this investigation was to study the behaviour of low-molecular-weight substances, giving special attention to those cases where interaction with the bed material occurs.

To make the following description more clear, the tested substances are divided into five groups. All experiments, however, are made on the same column, and the experimental results are quite comparable.

### 1. *Buffering substances*

The gel filtration method is a rapid and effective way of desalting solutions containing high-molecular-weight solutes. It can also be used for changing buffering ions in a protein solution[5].

The first experiments in this series were, therefore, made with those substances which can be used as buffers. With the intention of investigating the possibilities of using the method for desalting, most of the experiments were made with distilled water as an eluant (Table I).

All the tested substances except $\gamma$-collidine were eluted quantitatively with water. In some cases, a slight adsorption to the bed material occurs. This is most pronounced for sodium and potassium hydroxide and for sodium tetraborate, which are known to form complexes with carbohydrates. The strong adsorption of collidine cannot yet be explained, but collidine can, however, be quantitatively eluted from the column by an electrolyte solution.

### 2. *Amino acids*

Besides glycine, which is included in Table I, fourteen additional amino acids have been tested (Table II).

TABLE I

BUFFERING SUBSTANCES FILTERED THROUGH A COLUMN OF SEPHADEX G-25, 50–100 MESH

| *Substance* | *Quantity (mg)* | *Eluant* | $K_D$ | *Yield (%)* |
|---|---|---|---|---|
| Sodium hydroxide | 40 | Distilled water | 2.2 | 97 |
| Potassium hydroxide | 56 | Distilled water | 2.3 | 92 |
| Ammonium hydroxide | 34 | Distilled water | 0.8 | 93 |
| Sodium bicarbonate | 42 | Distilled water | 0.8 | 99 |
| Sodium carbonate | 29 | Distilled water | 0.8 | 103 |
| Sodium phosphate | 142 | Distilled water | 0.7 | 95 |
| Sodium tetraborate | 101 | Distilled water | 1.7 | 101 |
| Sodium acetate | 41 | Distilled water | 0.7 | |
| Sodium citrate | 129 | Distilled water | 0.5 | 99 |
| Hydrochloric acid | 18 | Distilled water | 0.8 | 94 |
| Formic acid | 23 | Distilled water | 0.9 | 88 |
| Acetic acid | 30 | Distilled water | 0.9 | 98 |
| Citric acid | 118 | Distilled water | 0.9 | 96 |
| Diethylbarbituric acid | 1.3 | Distilled water | 1.2 | 98 |
| Glycine | 0.8 | Distilled water | 0.9 | 91 |
| Triethylamine | 100 | Distilled water | 1.0 | |
| Pyridine | 1.5 | Distilled water | 1.2 | 97 |
| $\gamma$-Collidine | 1.5 | Distilled water | > 5 | |
| $\gamma$-Collidine | 1.5 | 0.05 *M* sodium chloride | 0.9 | 100 |

TABLE II

AMINO ACIDS FILTERED THROUGH A COLUMN OF SEPHADEX G-25, 50–100 MESH

| *Substance* | *Quantity (mg)* | *Eluant* | $K_D$ | *Yield (%)* |
|---|---|---|---|---|
| Alanine | 1.0 | Distilled water | 0.8 | 80 |
| Serine | 1.4 | Distilled water | 0.8 | 92 |
| Leucine | 2.4 | Distilled water | 0.8 | 94 |
| Methionine | 2.2 | Distilled water | 0.8 | 90 |
| Proline | 3.0 | Distilled water | 0.8 | 86 |
| Hydroxyproline | 4.8 | Distilled water | 0.8 | 97 |
| Phenylalanine | 2.5 | Distilled water | 1.0 | 91 |
| Tyrosine | 7.5 | Distilled water | 1.1 | 98 |
| Tryptophan | 2.1 | Distilled water | 1.9 | 91 |
| Aspartic acid | 2.4 | Distilled water | 0.2 | 83 |
| Aspartic acid | 1.6 | 0.05 *M* sodium chloride | 0.8 | 95 |
| Glutamic acid | 2.3 | Distilled water | 0.2 | 91 |
| Glutamic acid | 2.3 | 0.05 *M* sodium chloride | 0.8 | 98 |
| Lysine hydrochloride | 2.5 | Distilled water | > 3.3 | |
| Lysine hydrochloride | 2.5 | 0.05 *M* sodium chloride | 1.0 | 100 |
| Arginine hydrochloride | 3.0 | Distilled water | > 13 | |
| Arginine hydrochloride | 3.0 | 0.05 *M* sodium chloride | 1.0 | 97 |
| Histidine hydrochloride | 3.0 | Distilled water | > 3.6 | |
| Histidine hydrochloride | 3.0 | 0.05 *M* sodium chloride | 0.9 | 107 |

With distilled water as eluant, most of the amino acids are eluted with a $K_D$ about 0.8, which indicates a free diffusion in the column. $K_D$ does not reach the value of 1.0, because of the water of hydration. The aromatic amino acids show some adsorption to the bed material, and the basic amino acids are strongly adsorbed.

The acid amino acids, on the contrary, are partially excluded from the gel grains. Just as in the case of collidine, these anomalies disappear when the eluant contains an electrolyte.

### 3. *Nucleotides, nucleosides, purines, and pyridines*

Because of their great biological significance, some purine- and pyrimidine derivatives were tested on the gel column (Table III).

Heterocyclic substances, except for some of the nucleotides, are also adsorbed to the bed material. In water, the nucleotides are almost completely excluded from the gel grains, but this behaviour is normalized when the elution is done with an electrolyte solution.

TABLE III

PURINE AND PYRIMIDINE DERIVATIVES FILTERED THROUGH A COLUMN OF SEPHADEX G-25, 50–100 MESH

| *Substance* | *Quantity (mg)* | *Eluant* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Distilled water* | | *0.05 M sodium chloride* | | *Phosphate, $\mu = 0.05$, pH 7* | |
| | | $K_D$ | *Yield (%)* | $K_D$ | *Yield (%)* | $K_D$ | *Yield (%)* |
| Ribonucleic acid | 3.1 | 0.0 | 90 | | | | |
| Diphosphopyridine nucleotide | 2.8 | 0.0 | 100 | 0.8 | 81 | | |
| Adenosine triphosphate | 2.0 | 0.0 | 74 | 0.6 | 94 | | |
| Adenylic acid | 1.4 | 0.1 | 85 | 1.2 | 99 | | |
| Guanylic acid | 1.7 | 0.4 | 82 | 1.3 | 99 | 0.9 | 82 |
| Cytidylic acid | 1.9 | 0.1 | 82 | 0.8 | 96 | 0.7 | |
| Uridylic acid | 1.3 | 0.1 | 98 | 0.8 | 100 | 0.7 | 93 |
| Adenosine | 1.1 | 1.7 | 100 | 1.8 | | | |
| Guanosine | 1.3 | 1.6 | 93 | | | 1.8 | 82 |
| Cytidine | 1.3 | 1.2 | 93 | | | 1.2 | |
| Uridine | 1.2 | 1.0 | 94 | | | 1.0 | |
| Inosine | 1.2 | 1.2 | 96 | 1.3 | 101 | | |
| Adenine | 0.6 | 2.2 | 89 | 2.4 | 99 | | |
| Cytosine | 0.8 | 1.6 | 93 | | | 1.4 | |
| Uracil | 0.8 | 1.1 | 84 | | | 1.2 | |
| Hypoxanthine | 1.0 | 1.6 | 103 | 1.6 | 101 | | |
| Xanthine | 1.3 | 1.8 | 88 | | | | |
| Dimethylxanthine | 1.2 | 1.3 | 100 | | | | |
| Trimethylxanthine | 1.2 | 1.1 | 96 | | | | |

### 4. *Vitamins and alkaloids*

Table IV shows the results of experiments made with some vitamins and alkaloids.

As was the case for collidine and for the basic amino acids, the basic substances in this series of experiments are strongly adsorbed to the bed material in distilled water. When the elution is carried out with a solution containing an electrolyte, however, even those substances that are most strongly adsorbed in distilled water are recovered quantitatively, showing only a faint reversible adsorption.

TABLE IV

VITAMINS AND ALKALOIDS FILTERED THROUGH A COLUMN OF SEPHADEX G-25, 50–100 MESH

| *Substance* | *Quantity (mg)* | *Eluant* | $K_D$ | *Yield (%)* |
|---|---|---|---|---|
| Ascorbic acid | 1.4 | Distilled water | 0.9 | 103 |
| Riboflavin | 0.6 | Distilled water | 1.6 | 93 |
| Nicotinamide | 2.0 | Distilled water | 1.4 | 95 |
| Thiamine hydrochloride | 2.3 | Distilled water | $> 2.9$ | |
| Thiamine hydrochloride | 2.3 | 0.05 *M* sodium chloride | 1.0 | 91 |
| Pyridoxine hydrochloride | 1.6 | Distilled water | $> 3.3$ | |
| Pyridoxine hydrochloride | 1.6 | 0.05 *M* sodium chloride | 1.0 | 104 |
| Codeine hydrochloride | 10.1 | Distilled water | $> 3.5$ | |
| Codeine hydrochloride | 12.0 | 0.05 *M* sodium chloride | 1.2 | 99 |
| Morphine hydrochloride | 14.0 | Distilled water | $> 3.3$ | |
| Morphine hydrochloride | 12.1 | 0.05 *M* sodium chloride | 1.3 | 101 |
| Quinine sulphate | 11.9 | Distilled water | $> 2.9$ | |
| Quinine sulphate | 12.0 | 0.05 *M* sodium chloride | 1.6 | 102 |

### 5. *Some simple aromatic compounds*

From the preceding experiments, one can conclude that substances having an aromatic or heterocyclic structure as well as those of basic nature will cause some interaction with the bed material, resulting in delayed elution. The experiments with aromatic substances were extended with some additional examples, which are summarized in Table V.

TABLE V

AROMATIC SUBSTANCES FILTERED THROUGH A COLUMN OF SEPHADEX G-25, 50–100 MESH

| *Substance* | *Quantity (mg)* | *Eluant* | $K_D$ | *Yield (%)* |
|---|---|---|---|---|
| Benzoic acid | 5.0 | Distilled water | 0.5 | |
| Salicylic acid | 1.3 | Distilled water | 0.3 | 103 |
| Anthranilic acid | 2.6 | Distilled water | 0.6 | |
| Sulphanilic acid | 0.6 | Distilled water | 0.3 | 103 |
| Picric acid | 0.8 | Distilled water | 0.4 | 100 |
| Cinnamic acid | 0.3 | Distilled water | 0.3 | 109 |
| Phthalic acid | 6.0 | Distilled water | 1.1 | 98 |
| Salicylic acid | 2.0 | 0.05 *M* sodium chloride | 1.6 | 95 |
| Sulphanilic acid | 0.7 | 0.05 *M* sodium chloride | 1.1 | 103 |
| Picric acid | 0.6 | 0.05 *M* sodium chloride | 2.5 | 94 |
| Picric acid | 0.6 | Phosphate pH 7 | 2.7 | 97 |
| Phenol | 3.2 | Distilled water | 0.7 | 101 |
| Phenol | 4.0 | 0.05 *M* sodium chloride | 1.7 | 97 |
| Aniline | 4.0 | Distilled water | 1.5 | 98 |
| Benzyl alcohol | 52.5 | Distilled water | 1.3 | 95 |
| Salicyl alcohol | 3.9 | Distilled water | 1.4 | 103 |

These experiments indicate that all aromatic compounds are adsorbed to some extent to the bed material. The early elution of acids with water is once more established. This negative sorption is avoided when the elution is made with a salt solution.

### *Elution at various pH values*

In order to investigate the possibility of using acid and alkaline solutions as eluants, some experiments were made with elution at extreme pH values. It would also be of interest to see how a shift in pH might influence the sorption effects. Collidine, sulphanilic acid and tryptophan were chosen as representative substances.

TABLE VI

COLLIDINE, SULPHANILIC ACID, AND TRYPTOPHAN FILTERED THROUGH A COLUMN OF SEPHADEX G-25, 50–100 MESH AT DIFFERENT pH VALUES

| *Eluant* | *pH* | *Collidine 2.0 mg* | | *Sulphanilic acid 0.5 mg* | | *Tryptophan 2.0 mg* | |
|---|---|---|---|---|---|---|---|
| | | $K_D$ | *Yield (%)* | $K_D$ | *Yield (%)* | $K_D$ | *Yield (%)* |
| 0.01 *M* hydrochloric acid | 2.0 | 0.8 | 101 | 0.9 | 93 | 2.2 | 100 |
| 1.0 *M* acetic acid | 2.4 | 0.8 | 99 | 0.9 | 100 | 2.0 | 91 |
| 0.02 *M* phosphate | 7.0 | 1.0 | 101 | 1.1 | 100 | 2.1 | 100 |
| 0.05 *M* triethylammonium carbonate | 9.0 | 1.5 | 98 | 0.9 | 100 | 1.7 | 96 |
| 0.01 *M* ammonium hydroxide | 10.6 | 1.5 | 86 | 0.4 | 99 | 0.5 | 96 |
| 0.01 *M* sodium hydroxide | 12.0 | 1.5 | 93 | 0.5 | 99 | 0.7 | 99 |

TABLE VII

SOME AROMATIC AND HETEROCYCLIC SUBSTANCES FILTERED THROUGH A COLUMN OF SEPHADEX G-25, 50–100 MESH, WITH 0.01 *M* AMMONIUM HYDROXIDE pH 10.6 AS ELUANT

| *Substance* | *Quantity (mg)* | *Ammonium hydroxide* | | *Sodium chloride* |
|---|---|---|---|---|
| | | $K_D$ | *Yield (%)* | $K_D$ |
| Adenylic acid | 1.4 | 0.1 | 96 | 1.2 |
| Guanylic acid | 1.3 | 0.1 | 97 | 1.3 |
| Cytidylic acid | 1.6 | 0.1 | 96 | 0.8 |
| Uridylic acid | 1.5 | 0.1 | 95 | 0.8 |
| Picric acid | 0.5 | 0.4 | 98 | 2.5 |
| Salicylic acid | 2.2 | 0.5 | 101 | 1.6 |
| Adenosine | 1.0 | 1.8 | 100 | 1.8 |
| Cytosine | 0.7 | 1.3 | 99 | |
| Inosine | 1.2 | 0.3 | 100 | 1.3 |
| Adenine | 0.5 | 1.2 | 98 | 2.4 |
| Phenol | 3.7 | 1.0 | 100 | 1.7 |
| Benzyl alcohol | 50.0 | 1.3 | 96 | |
| Riboflavin | 0.8 | 0.8 | 88 | |
| Nicotinamide | 2.1 | 1.3 | 100 | |
| Thiamine hydrochloride | 1.5 | 0.3 | 95 | 1.0 |
| Pyridoxine hydrochloride | 1.6 | 0.7 | 98 | 1.0 |

It is evident that one gets a quantitative recovery over the whole pH range, and that the sorption properties of the bed material varies with pH.

It is interesting to find that the sorption properties are so drastically changed at high pH values. The negative sorption of acid substances is more accented and the adsorption of aromatic substances seems to be decreased. The experiments with alkaline solution (0.01 $M$ ammonium hydroxide) as eluant were therefore extended to include other aromatic and heterocyclic substances.

For comparison, $K_D$ values from experiments in 0.05 $M$ sodium chloride are included in Table VII.

The exclusion of acid substances on elution with distilled water or alkaline solution is valid only when small quantities are filtered through the column. For large quantities, the main portion of the acid is eluted in a normal way, *i.e.* with the same $K_D$ as when an electrolyte solution is used as eluant. However, in these cases, too, the elution starts too early, and the zone is spread out in a large effluent volume. This is illustrated in Fig. 3a, where 50 mg of picric acid is filtered through the column with water. In the corresponding experiment in Table V, the picric acid had $K_D = 0.4$, and 100 % of the acid was eluted within an effluent volume equal to the volume $V_o + V_i$. In this case, the $K_D$ value is 1.6, and only 6.5 % of the applied acid is eluted within the same volume. Analogous experiments have been made with sulphanilic acid and salicylic acid with distilled water as well as diluted ammonium hydroxide as eluant, and similar results were obtained.

A similar quantitative limitation has also been found for those basic substances which are strongly adsorbed to the bed material on elution with distilled water. Experiments have been made with frontal analysis of collidine and alkaloids, but the capacity of the column for these substances has not been determined.

*Filtration experiments with mixtures of low-molecular-weight substances*

In all the previous experiments, the tested substances are separately filtered through the column. We shall now discuss some experiments made with mixtures of these test substances. The first example relates to the experiments reported in Table VI.

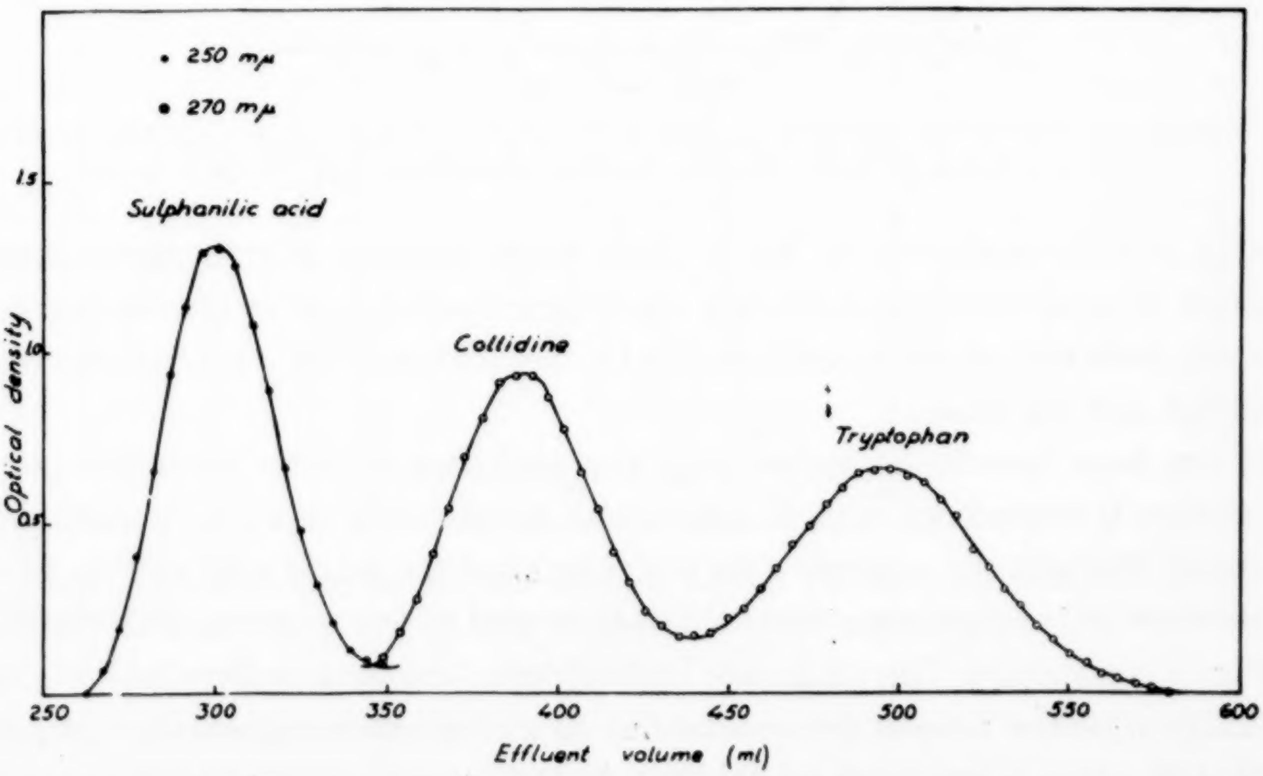

Fig. 1. 0.6 mg sulphanilic acid, 2.0 mg collidine, and 2.0 mg tryptophan filtered through a column (3.5 × 35 cm) of Sephadex G-25, 50–100 mesh. Eluant: 0.05 $M$ triethylammonium carbonate pH 8.0.

At pH 8–9 it should be possible to separate the three substances: sulphanilic acid, collidine, and tryptophan. Fig. 1 shows the elution diagram when the eluant is 0.05 *M* triethylammonium carbonate at pH 8.0. The $K_D$ values 0.8, 1.4, and 2.0 correspond well to those obtained for the three substances in separate experiments.

Some diagrams for purine and pyrimidine derivatives eluted with phosphate buffer ($\mu = 0.05$) are given in Fig. 2. The third diagram in this figure shows the separation of a mixture corresponding to an acid hydrolysate of ribonucleic acid. In agreement with the results listed in Table III, separation is only possible between the nucleotides and the bases.

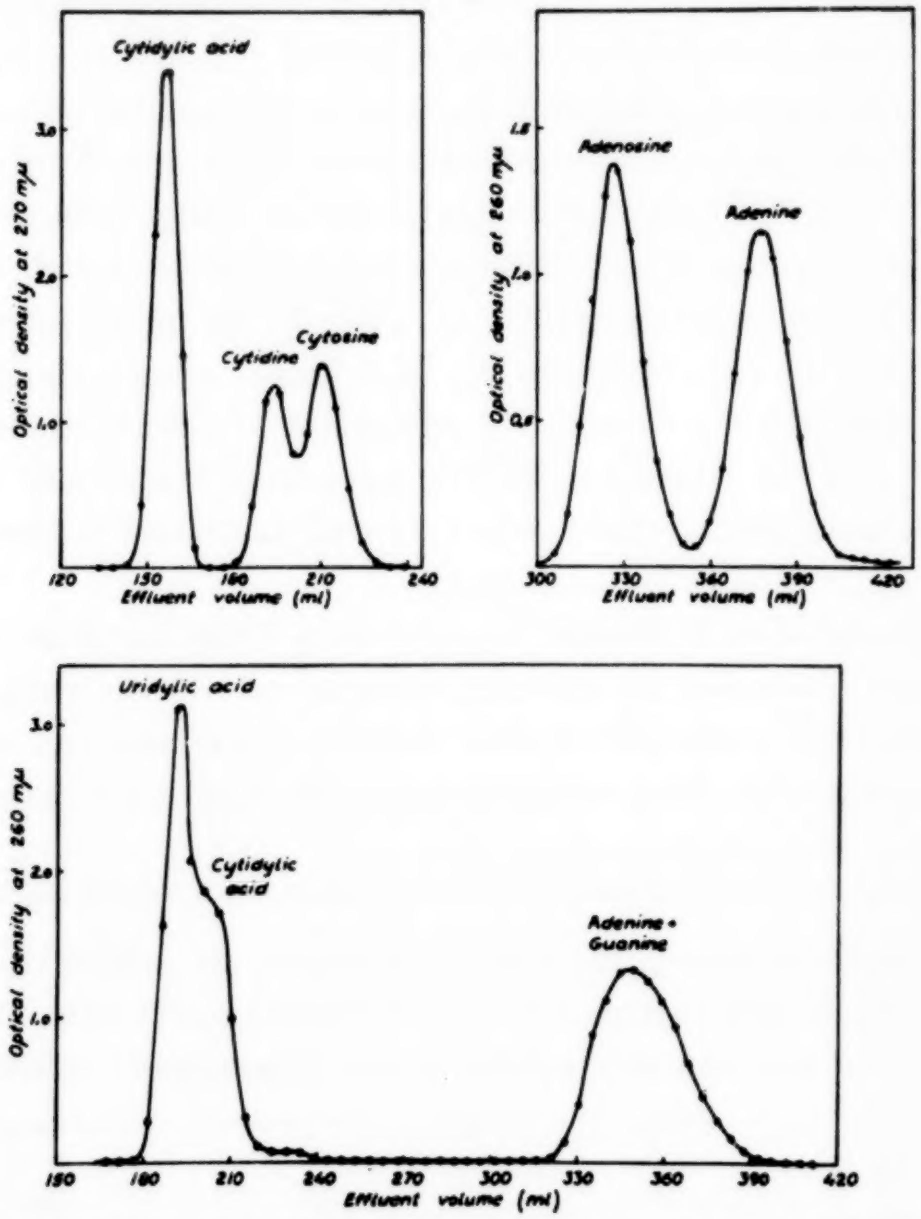

Fig. 2. Purine and pyrimidine derivatives filtered through a column (3.0 × 30 cm) of Sephadex G-25, 200–400 mesh. Eluant: Sodium phosphate, pH 7.0 ($\mu = 0.05$).

It has been mentioned earlier that the exclusion of acids from the gel grains when elution is carried out with distilled water is valid only when very small amounts are filtered through the column. This is exemplified for picric acid in Fig. 3a. If the test solution contains 25 mg sodium chloride as well as 50 mg picric acid, the diagram b in Fig. 3 is obtained. The peaks are hydrochloric acid and sodium picrate, respectively. This time the zone is not spread out as in the preceding experiment, but the picrate will attain a partition equilibrium in the inner and outer water phase when passing through the column. A similar result is also obtained with collidine when the

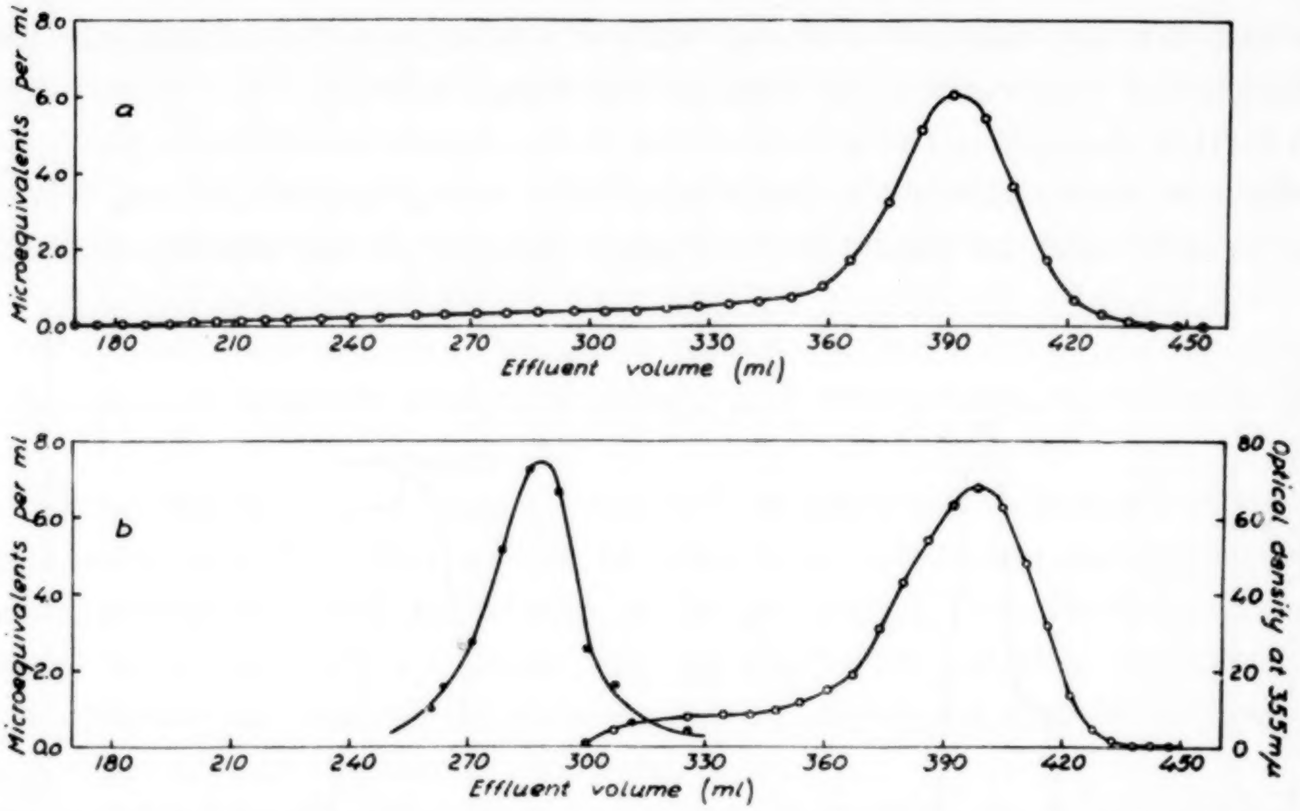

Fig. 3. (a) 50 mg picric acid (b) 50 mg picric acid and 25 mg sodium chloride filtered through a column (3.5 × 35 cm) of Sephadex G-25, 50–100 mesh. Eluant: Distilled water. ○ picrate; ● chloride ions.

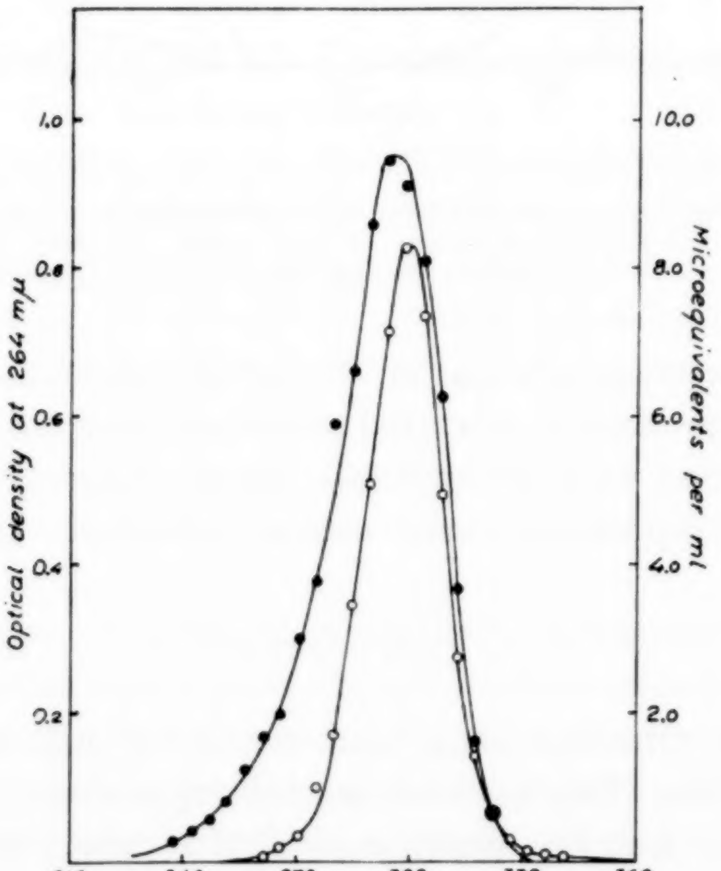

Fig. 4. 3 mg collidine and 25 mg sodium chloride filtered through a column (3.5 × 35 cm) of Sephadex G-25, 50–100 mesh. Eluant: Distilled water. ○ collidine; ● chloride ions.

test solution contains an electrolyte. Fig. 4 shows an experiment where 3 mg collidine and 25 mg sodium chloride are washed through the column with water. Usually the collidine is strongly adsorbed to the bed material, but, in the presence of an electrolyte in the test solution, collidine is eluted without any appreciable adsorption, just as if the elution had been carried out with an electrolyte solution.

A further example of the normalization of the elution conditions is given in Fig. 5. A mixture of 20 mg glycine, 20 mg methionine, 20 mg tyrosine, 20 mg tryptophan, 20 mg aspartic acid, 20 mg lysine hydrochloride, and 20 mg arginine hydrochloride dissolved in 15.0 ml of water was put on the column and eluted with distilled water. All the aliphatic amino acids were eluted simultaneously with a $K_D$ about 1, which is comparable to elution with electrolyte solution (Table II). The aromatic amino acids, tyrosine and tryptophan, were weakly adsorbed. The total recovery was quantitative.

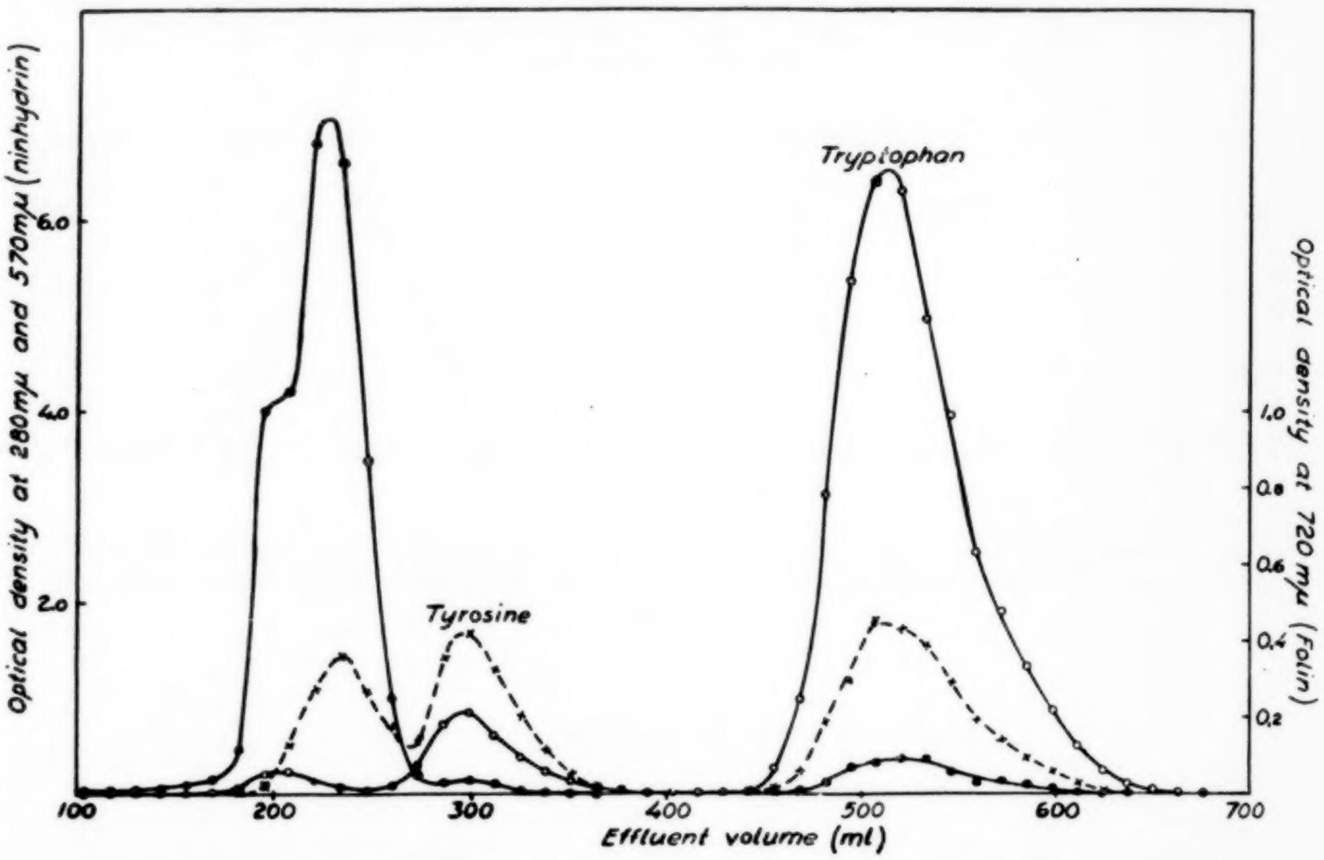

Fig. 5. 20 mg glycine, 20 mg methionine, 20 mg tyrosine, 20 mg tryptophan, 20 mg aspartic acid, 20 mg lysine hydrochloride, and 20 mg arginine hydrochloride in 15.0 ml of water filtered through a column (3.5 × 40 cm) of Sephadex G-25, 50–100 mesh. Eluant: Distilled water. o Optical density at 280 mμ; • 570 mμ (ninhydrin reagent); x 720 mμ (Folin-Ciocalteu reagent).

## CONCLUSIONS

The main effect in gel filtration using Sephadex as bed material is the separation of molecules of different size. This has been convincingly shown in other papers[1,2,5–7]. However, the data given in this paper show that a variety of other factors may influence the result of an experiment. These factors are most pronounced for low-molecular-weight substances, but in some cases they may also be relevant for substances of high molecular weight.

For an adequate discussion and a better understanding, it seems reasonable to separate these secondary effects into two groups.

*A*. Adsorption to the bed material, which is related to the structure of the test substances and is largely independent of the properties of the solvent.

*B*. Superimposed effects, which depend on the conditions of the column, *i.e.* the ionic strength and pH of the aqueous phases.

*A*. Among the inorganic ions tested, hydroxyl and borate ions are adsorbed. However, the experimental material is small, and there may be other ions which interact strongly with the gel matrix. For organic compounds, aromatic and heterocyclic substances have a greater tendency to be adsorbed than aliphatic substances. Basic groups in a molecule seem to increase, and acid groups to decrease, the adsorption.

*B*. These effects appear when a column is equilibrated with distilled water and are manifested in a strong adsorption of some basic substances and an exclusion of some acid substances from the interior of the gel grains. The effects are completely eliminated when the elution is made with an electrolyte solution, or when the test solution contains an electrolyte. However, an exclusion of acid substances is also obtained when elution is made at $\mathrm{pH} > 10$.

There is also another distinction between the two groups *A* and *B*. The effects in *B* are active only for very small quantities of test substance, while the adsorption of aromatic and heterocyclic substances (effect in *A*) is obtained for much larger quantities, giving symmetrical peaks which indicate linear adsorption isotherms. This circumstance and the observations mentioned above make it appear that the effects under *B* are caused by the small amount of ionized carboxylic groups in the bed material.

With these facts in mind, one can predict the result of a gel filtration experiment even with a rather complex mixture. There is always a slight retention of aromatic and heterocyclic substances. Only in very few cases will there be a strong adsorption of some basic compounds and a negative sorption of acid compounds, *i.e.* when the experiment is made in complete absence of other ionized substances. If other ionized substances are present, or if the elution is made with an electrolyte solution, these particular sorption effects disappear. The best way to avoid these superimposed effects is to perform the experiments in a medium containing at least a small amount of electrolyte.

There may, however, be instances when the secondary effects are of great value. Figs. 1, 2 and 5 are examples of a pure adsorption chromatography. A combination of adsorption chromatography and the molecular sieve effect is also of interest[5].

It is assumed that low-molecular-weight substances with $K_D$ values about 0.8 are those which pass the column without sorption and that a correction of the inner volume $V_i$ for the water of hydration would give $K_D = 1$. Also in batch experiments with some of these substances $K_D = 0.8$ is obtained over a wide concentration range[2].

In spite of the secondary effects discussed in this paper, Sephadex seems to be very suitable as column material in gel filtration. None of the tested substances is irreversibly adsorbed to the column, recoveries are almost quantitative in all experiments, and the reproducibility is fairly good. Furthermore, the mechanical and chemical stability of the bed material is quite satisfactory.

The majority of experiments reported in this paper are made on the same column without any detectable change in its properties.

## SUMMARY

1. The principle of the gel filtration method is given, and experiments with buffering substances, amino acids, purine and pyrimidine derivatives, vitamins, alkaloids and some simple, aromatic substances are described.

2. The data presented demonstrate a good reproducibility. $K_D$ values and recoveries are given in almost all experiments.

3. The experiments show that the bed material, Sephadex, in addition to the molecular sieve effect, gives a slight adsorption of aromatic and heterocyclic substances.

4. Depending upon the ionic strength and the pH of the gel column, a strong adsorption of some basic substances and a negative sorption of some acidic substances may occur. These effects are easily eliminated by the proper choice of experimental conditions.

5. The column material Sephadex is very useful for gel filtration. It does not seem to give any irreversible adsorption, and more than one hundred experiments have been performed on the same column without any detectable change in its properties.

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# A SOLVENT FOR QUALITATIVE AND QUANTITATIVE DETERMINATION OF SUGARS USING PAPER CHROMATOGRAPHY

P. COLOMBO, D. CORBETTA, A. PIROTTA, G. RUFFINI AND A. SARTORI
*Vita Mayer & Co., Research Division, Milan (Italy)*

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## QUALITATIVE ANALYSIS

In carrying out studies of the carbohydrate fraction of wood[1], we have found it necessary to have a rapid and accurate method for the separation and qualitative and quantitative determination of the five principal sugars constituting this fraction: glucose, galactose, mannose, arabinose and xylose.

The classical methods of analysis, while adequate for the determination of a single sugar, are not suitable for the determination of a mixture. In fact, neither the technique of precipitation with specific reagents nor the method of selective sugar fermentation can be used to solve the problem of the separation of the five sugars.

PARTRIDGE's method[2] of paper chromatography adapted from the method for the determination of amino acids[3], overcomes some of the difficulties, mainly by the simplicity of its technique. In fact many workers have used chromatographic analysis for the study of the hemicelluloses and of the composition of analytic $\alpha$-cellulose. We can also say that, although this method is little used at present, it can give good results in the study of the composition of wood cooking liquors.

The method of paper chromatography did not previously completely solve the problem of the separation of sugar mixtures; in fact practically none of the eluent solvents used by other workers[4-7] gave a satisfactory result when used for a complete and rapid separation of the five sugars in the mixture. The solvents commonly used are based on butanol: the acid ones separate mannose from arabinose but not glucose from galactose; the basic ones separate glucose from galactose but not arabinose from mannose. For quantitative analysis it would be necessary to make two successive elutions using both acid and basic solvents.

To achieve a good separation, BOGGS[8] employed a double elution method. Such a determination, carried out at room temperature, requires about 40 hours, which we consider an excessive amount of time.

The purpose of this work was to find a solvent whose use would make possible the separation of monosaccharides from mixtures in reasonable time.

We began by examining numerous solvents suggested by various authors using the method of descending and ascending paper chromatography. For all the solvents we examined, we found that the method of descending chromatography produced better separation than the ascending method, although the spots were somewhat elongated.

Furthermore, for all the solvents we found that better separation of monosaccharides is obtained using the system of multiple elutions: this consists of repeatedly drying and eluting the paper with fresh eluent.

The advantages obtained by using this method, which requires constant attention, are not such as to make it preferable to the normal elution process even though the time consumed is almost the same; this became more obvious when the ascending method was used.

In fact, in this case an increase of elution time over that required for the eluent to reach the top of the sheet is useless as any increase in time results in the production of diffused spots with lateral elongation.

A series of acid and basic eluents based on butanol, were tried using different proportions of: butanol–acetic acid–water, and butanol–pyridine–water.

The migration velocities of the monosaccharides were increased by substituting ethyl alcohol for the acid or the base, but the separation was still unsatisfactory.

This type of solvent gave good results when used for the separation of oligosaccharides, but not with monosaccharides, where the velocity of migration is too great.

We examined the possibilities of low viscosity solvents based on ethyl acetate, while realizing that double phase solvents give better results than monophase solvents.

The solvent mixture consisting of ethyl acetate, acetic acid and water gave fairly good results for the separation of mannose and arabinose; the separation of glucose and galactose was far better than with other solvents, but still unsatisfactory.

A basic mixture, ethyl acetate, pyridine and water gave the best results.

Using this mixture in the proportions: 3.6:1:1.15 and the descending method we obtained a complete separation of five principal sugars with elution times varying from 18 to 25 h at room temperature (about 20°). Fig. 1 shows a chromatogram of a mixture of six sugars obtained in 18 h elution at a laboratory temperature of 20°. Very good reproducibility of results was obtained without taking any special precaution to maintain constant conditions.

At 30°, a very good chromatogram was obtained in 15 h: naturally at this temperature we have to use a thermostatically controlled oven, but the spots are of good shape: round and compact, without tails after development.

Diffused spots and tailing can also be partially avoided by cutting the paper exactly perpendicular to the main fiber direction and then carrying out the chromatography at room temperature, as it was done for the chromatogram in Fig. 1.

Good results as regards roundness and compactness of spots can also be obtained by cutting the paper obliquely to the main fiber direction.

The presence of mineral salts ($Na_2SO_4$) does not influence the distinct separation of various monosaccharides with the solvent used, although the spots may be somewhat less regular and compact.

The best separations were obtained using drops of 2 µl of sugar solution at a concentration of each monosaccharide varying from 10 µg/µl to 2 µg/µl.

After elution and drying we used the modified PARTRIDGE'S developing agent consisting of acid aniline phthalate in water-saturated butanol. The solution was freshly prepared before each determination, thus preventing any discoloration of the solution and obtaining maximum contrast between the spots and the paper.

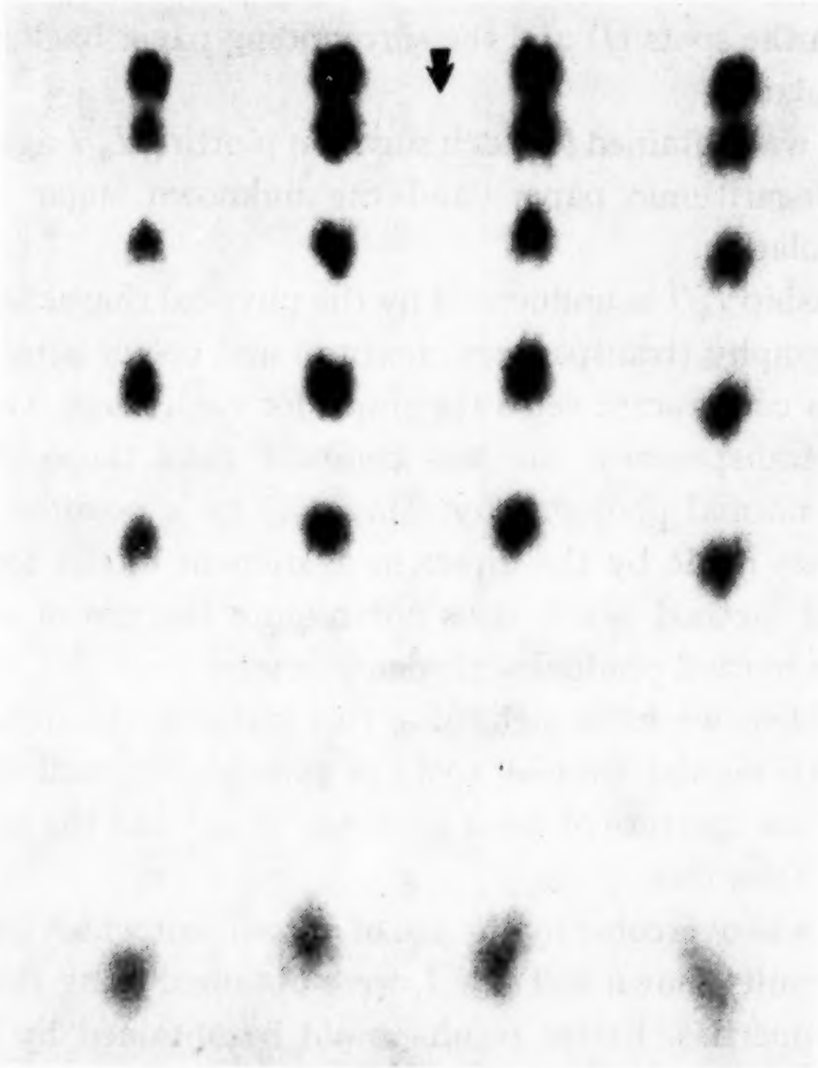

Fig. 1. Chromatogram of a mixture of six sugars. Elution time at 20° = 18 h.

## QUANTITATIVE ANALYSIS

After overcoming, by the use of our solvent, the problem of the distinct and rapid separation of the monosaccharides, we turned our attention to the possibilities of quantitative determinations.

Using a micropipette, we placed a 2 µl drop of the unknown sugar solution at two places on the departure line of the chromatography sheet; at five other places we placed 2 µl drops of solution of known sugar content. The process of chromatography was carried out as described in the experimental section.

The five drops of known solutions, containing the monosaccharides in various concentrations, were used to obtain a rough approximation of the concentration of the sugars in an unknown solution. After this previous observation, calibration solutions were prepared for the quantitative analysis.

For every single sugar five spots were obtained, whose colour intensity was directly proportional to the sugar concentration.

We preferred the method of direct measurement of the total colour density of the spots.

After development and drying (oven at 105°), intensity measurements were carried out on both the spots ($I$) and the surrounding paper background ($I_0$) and the ratio $I_0/I$ was calculated.

A straight line was obtained for each sugar on plotting $I_0/I$ against concentration ($\mu$g/2 $\mu$l) on semi-logarithmic paper; and the unknown sugar concentration was obtained by interpolation.

As the relationship $I_0/I$ is unfluenced by the physical characteristics of the paper used for chromatography (transparency, texture) and colour after the development, it was necessary to construct a separate graph for each sheet. Generally, the spots photographed by transparency, are less compact than those observed by direct observation or by normal photography. This may be a possible cause of errors in quantitative analysis made by the direct measurement of the total colour density.

This is a rapid method which does not require the use of an apparatus more complicated than a normal photoelectric densitometer.

The main problem we have met using this meter is the difficulty in obtaining chromatograms with regular circular spots of sufficiently small dimensions. In fact the search unit has an aperture of fixed diameter (2 cm) and the spots must not have a greater diameter than this.

This difficulty was overcome by the use of our solvent which gave good resolution of the spots. The results shown in Table I, were obtained using this solvent and thus demonstrate its properties. Better results could be obtained by the use of a more accurate light meter.

It was also necessary to spray the developer as uniformly as possible and carry out the colour density measurements as soon as possible after development. In fact the sheets, after development and drying, have a tendency to become yellow on exposure to the air.

In order to establish the precision of the method, 12 chromatograms were prepared with a descending method, using the following solution of known composition (A):

| | |
|---|---|
| Glucose | 200 mg/100 ml |
| Galactose | 250 mg/100 ml |
| Mannose | 250 mg/100 ml |
| Arabinose | 250 mg/100 ml |
| Xylose | 750 mg/100 ml |

Each chromatogram was prepared by placing on the departure line, as mentioned before, 2 drops each of 2 $\mu$l of solution A and 5 drops of the standard calibration solutions.

The values for each single sugar obtained from the two drops were averaged. The sugar content given by each spot of solution A varied from 4 $\mu$g of glucose to 15 $\mu$g of xylose. The results obtained are given in Table I.

## TABLE I

PRECISION OF THE METHOD. RESULTS FROM 12 ANALYSES

| *Test* | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* | *9* | *10* | *11* | *12* | *Average value* | *Standard deviation* | *% Deviation from theory* | *Coefficient of variation* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Glucose | 195 | 195 | 200 | 190 | 195 | 205 | 205 | 205 | 205 | 200 | 200 | 210 | 200.41 | ± 5.79 | + 0.205 | ± 2.89 |
| Galactose | 255 | 240 | 240 | 250 | 237 | 265 | 245 | 240 | 260 | 250 | 250 | 250 | 248.50 | ± 8.60 | — 0.600 | ± 3.46 |
| Mannose | 245 | 255 | 250 | 250 | 240 | 250 | 250 | 235 | 250 | 250 | 235 | 270 | 248.33 | ± 9.34 | — 0.670 | ± 3.76 |
| Arabinose | 256 | 245 | 250 | 248 | 248 | 245 | 250 | 240 | 240 | 250 | 240 | 250 | 246.90 | ± 5.01 | — 0.840 | ± 2.03 |
| Xylose | 738 | 760 | 760 | 725 | 750 | 750 | 730 | 730 | 760 | 737 | 740 | 750 | 744.16 | ± 12.57 | — 0.780 | ± 1.69 |

Calculation of coefficient of variation:

$$S^2 = \frac{\Sigma\,(x - \bar{x})^2}{N - 1}\,.$$

$\Sigma\,(x - \bar{x})^2$ = sum of squared deviation of sample mean.

$\bar{x}$ = mean.

$\frac{100 \cdot S}{\bar{x}}$ = coefficient of variation.

$N$ = number of tests.

## EXPERIMENTAL DETAILS

Standard laboratory apparatus was used throughout this work. Chromatography was carried out in a glass air-tight vessel of dimensions 20 × 20 × 60 $cm^3$.

The troughs holding the eluent were also of glass and supported by steel uprights.

The chromatography paper used was Whatman No. 1 grade; and in each case the sheets were cut to the dimensions 22 × 46 $cm^2$.

The Photovolt densitometer Model 610 with a Blue Filter Wratten 49 was used to measure the colour density of the spots.

A descending method of chromatography was employed.

The drops of the sugar solutions were placed with a 2 $\mu$l micropipette along a pencil line, drawn across the width of the sheet at 10 cm from one end.

The sheets were introduced into the chromatography vessel and weighted by fastening to the ends of the sheets glass weights which served to increase the rate of flow of the solvent through the paper.

The solvent was freshly prepared by shaking, in a separating funnel, ethyl acetate, pyridine, water in the proportion 3.6:1:1.15. The mixture was allowed to separate into two layers: the lower layer was placed in the bottom of the chromatography tank to saturate the atmosphere. The upper layer was placed in the elution troughs.

The sheets after elution, which could take from 18 h (qualitative analysis) to 25 h (quantitative analysis) at a temperature of about 20°, were taken from the tank and dried at room temperature.

The sheets were developed by spraying, as uniformly as possible with a fine spray of developing solution under pressure from a nitrogen cylinder.

The developer was freshly prepared by dissolving 2.5 g of acid aniline phthalate in 100 ml of water-saturated butanol.

The acid aniline phthalate was prepared by dissolving phthalic acid in warm alcohol and then adding aniline. The solution was cooled and the precipitated acid aniline phthalate was recrystallized from alcohol and washed with ether.

By preparing the developer in this way the inconvenience of discoloration due to the presence of aniline impurities, which cannot be completely removed by distillation, was overcome.

The chromatography sheets, after spraying and development, were dried in an oven, in which the air was circulated, for a period of 5 min at 105° ± 1°.

Measurement of spot colour density, using a Photovolt densitometer, was carried out immediately after the spraying.

For a development time of 22 h (at a room temperature of about 20°) the movement of single sugars relative to the movement of glucose ($R_{Glu}$) was measured; the results are given below:

Glucose = 1.00
Galactose = 0.80
Mannose = 1.30
Arabinose = 1.64
Xylose = 2.18
Rhamnose = 4.30

The reproducibility of these results was found to be good and independent of room conditions.

Comparing these values with those given in the literature for other solvents a definite improvement in the separation was noted.

For 18 h elution time (always at room temperature) the $R_{Glu}$ values were somewhat modified:

Glucose = 1.00
Galactose = 0.80
Mannose = 1.30
Arabinose = 1.62
Xylose = 2.14

Experiments were also carried out at 30°, by placing the chromatography vessel in a thermostatically controlled oven.

In this case the initial spot of 2 $\mu$l contained 360 $\mu$g of xylose, 15 $\mu$g of galactose, 28 $\mu$g of glucose, 22 $\mu$g of mannose, 10 $\mu$g of arabinose and 7.5 $\mu$g of rhamnose. The $R_{Glu}$ values were calculated for 15 h elution time:

Glucose = 1.00
Galactose = 0.79
Mannose = 1.30
Arabinose = 1.57
Xylose = 2.01
Rhamnose = 3.16

The initial spots of sugars solutions of 2 $\mu$l and which contained every sugar in the mixture, in quantities varying from 40 $\mu$g to 8 $\mu$g, gave the best separations.

The rhamnose, traces of which were definitely found in the hydrolysate of poplar sawdust, has been introduced in some calibration tests to study the possibility of its quantitative determination, in the presence of large quantities of other sugars.

## SUMMARY

The object of this work was to find a rapid and precise method for the separation of the principal monosaccharide constituents of the carbohydrate fraction of wood: glucose, galactose, mannose, arabinose and xylose.

Discarding the imprecise methods of classical analysis, we have considered the possibility of the application of paper chromatographic methods.

None of the eluents suggested in the literature gives a satisfactory solution to this problem.

We have studied the use of a solvent composed of a mixture of ethyl acetate, pyridine and water in the proportions 3.6:1:1.15; we have found that this eluent gives a complete separation of the sugars and allows both qualitative and quantitative determinations.

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# ZUR GASVERTEILUNGSCHROMATOGRAPHIE STEREOISOMERER MENTHOLE

H. J. PETROWITZ, F. NERDEL UND G. OHLOFF*

*Organisch-chemisches Institut der Technischen Universität, Berlin und Laboratorium der Firma DRAGOCO, Holzminden (Deutschland)*

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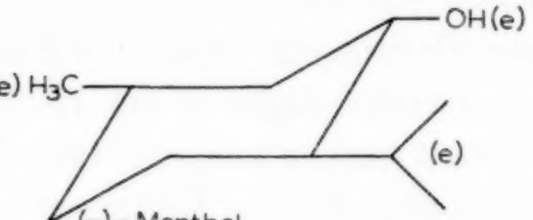
Die drei asymmetrischen Kohlenstoff-Atome im Menthol bedingen 8 optisch aktive Stereoisomere bzw. 4 Racemate, deren Konformationen[1], bekannt sind. Technische Bedeutung besitzt lediglich die *l*-Menthol-Reihe, der das pharmakologisch wichtige (—)-Menthol (I) angehört. Zur Darstellung von (—)-Menthol lässt man stufenweise oder in einem Reaktionsschritt verschiedene Reduktionsmittel auf optisch aktives Piperiton, Pulegon oder (—)-Menthon (V) und (+)-Isomenthon (VI) einwirken, wobei in wechselnder Menge die 4 Stereoisomeren I–IV entstehen.

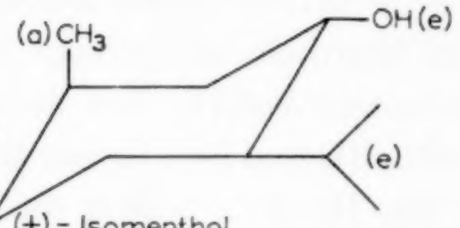
Die Bestimmung der isomeren Menthole in ihren Gemischen bereitet grosse Schwierigkeiten. Kortüm und Bittel[2] berichten über ein Verfahren, zur Gegenstromverteilung in einer Scheibelkolonne — Lösungsmittelphasen: Heptan–Wasser/Methanol—zur Trennung der Stereoisomeren I–III. Danach gab das Gemisch aus (+)-Neomenthol(II) und (—)-Menthol(I) (1:1) bei einmaligem Durchgang eine

I



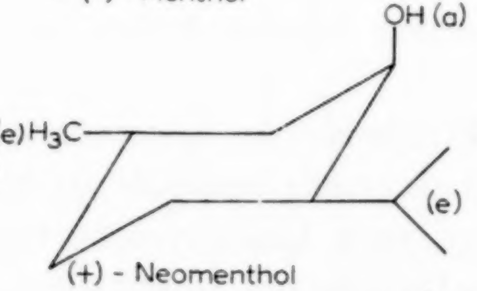
(−) - Menthol

III



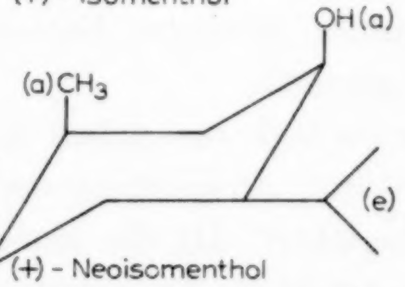
(+) - Isomenthol

II



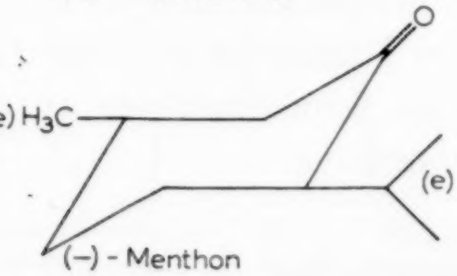
(+) - Neomenthol

IV



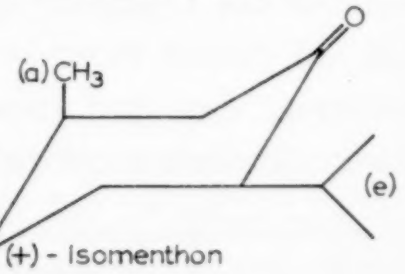
(+) - Neoisomenthol

V



(−) - Menthon

VI



(+) - Isomenthon

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* Neue Anschrift: Max Planck-Institut für Kohlenforschung, Abt. Strahlenchemie, Mühlheim a. d. Ruhr.

TABELLE I

RETENTIONSZEITEN STEREOISOMERER MENTHOLE

| | *Spalte 1* | *Spalte 2* | | *Spalte 3* |
|---|---|---|---|---|
| | *Säule A, 2 m*<br>*180°* | *Säule A, 4 m*<br>*180°* | | *Säule A, 4 m*<br>*160°* |
| | *Substanz ungelöst* | *Substanz ungelöst* | *Substanz in Aceton gelöst* | *Substanz ungelöst* |
| Neomenthol | 25′50″ | 54′20″ | 54′10″ | 72′0″ |
| Neoisomenthol | — | — | 57′20″ | 80′30″ |
| Menthol | 27′30″ | 59′30″ | 57′30″ | — |
| Isomenthol | 29′10″ | 62′50″ | 60′30″ | 82′50″ |

praktisch quantitative Trennung, während (—)-Menthol (I) und (+)-Isomenthol (III) in beiden Lösungsmittelphasen nur bis zu einem gewissen Grade angereichert werden. Die Verteilung der epimeren Menthole hängt hierbei im wesentlichen von ihrer Polarität ab. Neoisomenthol (IV) wurde in diese Untersuchungen nicht einbezogen.

Eine teilweise Auftrennung der Stereoisomeren Menthole gelang Hückel und Mitarb.[3,4] durch Adsorptionschromatographie entsprechender Gemische an $Al_2O_3$ (Woelm, Aktivität 4). Eine Trennung wurde hier lediglich bei Gemischen aus Isomenthol und Neoisomenthol erreicht. Menthol und Isomenthol sind nach diesem Verfahren praktisch nicht trennbar. Demnach scheint es so, dass hierbei nur die epimeren Menthole mit axialer Stellung der Hydroxylgruppe, (+)-Neomenthol(II) und Neoisomenthol(IV) von denen mit äquatorialer Stellung der Hydroxylgruppe, (—)-Menthol(I) und (+)-Isomenthol (III)getrennt werden können. Nach den Beobachtungen von Hückel besitzt die erste Gruppe eine höhere Elutionsgeschwindigkeit als die zweite. Ähnliche Beobachtungen wurden bereits früher an 3-Hydroxysteroiden[5] gemacht.

In der vorliegenden Arbeit wird über die Ergebnisse der Trennung stereoisomerer Menthole durch Anwendung der Gasverteilungschromatographie nach James und Martin[6] berichtet. Da die einzelnen Isomeren zwar keine grossen aber immerhin deutlich feststellbare Dampfdruckunterschiede* aufweisen, und da ihre Verteilungskoeffizienten auf Grund ihrer konformationellen Unterschiede ebenfalls differieren, war zu erwarten, dass mit dieser Technik eine Schnellmethode zur Analyse des stereoisomeren Gemisches entwickelt werden könnte.

## VERFAHREN UND ERGEBNISSE

Die Untersuchungen wurden mit einem Fraktometer der Firma Perkin-Elmer, Bodenseewerk Überlingen (Modell 116) durchgeführt. Die Wahl der stationären Phase wurde in Anlehnung an die Vorschläge von Raupp[12] vorgenommen. Danach erschien uns Di-*n*-decylphthalat (Säule A von Perkin-Elmer) als stationäre Phase zur Lösung unseres Problems geeignet. Tatsächlich erzielten wir mit dieser Säule bessere Ergebnisse als mit Diäthyl-hexylsebacinat (Säule B), Siliconöl (Säule C) und Polyäthylenglykol (Säule K). Als günstigste Arbeitstemperatur wurde 180° ermittelt.

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* Neomenthol, K.p. 211.5–212.5°[7,8]; Neoisomenthol, K.p. 214–215°[9,10]; Menthol, K.p. 216.5°[11]; Isomenthol, K.p. 218.6°[11].

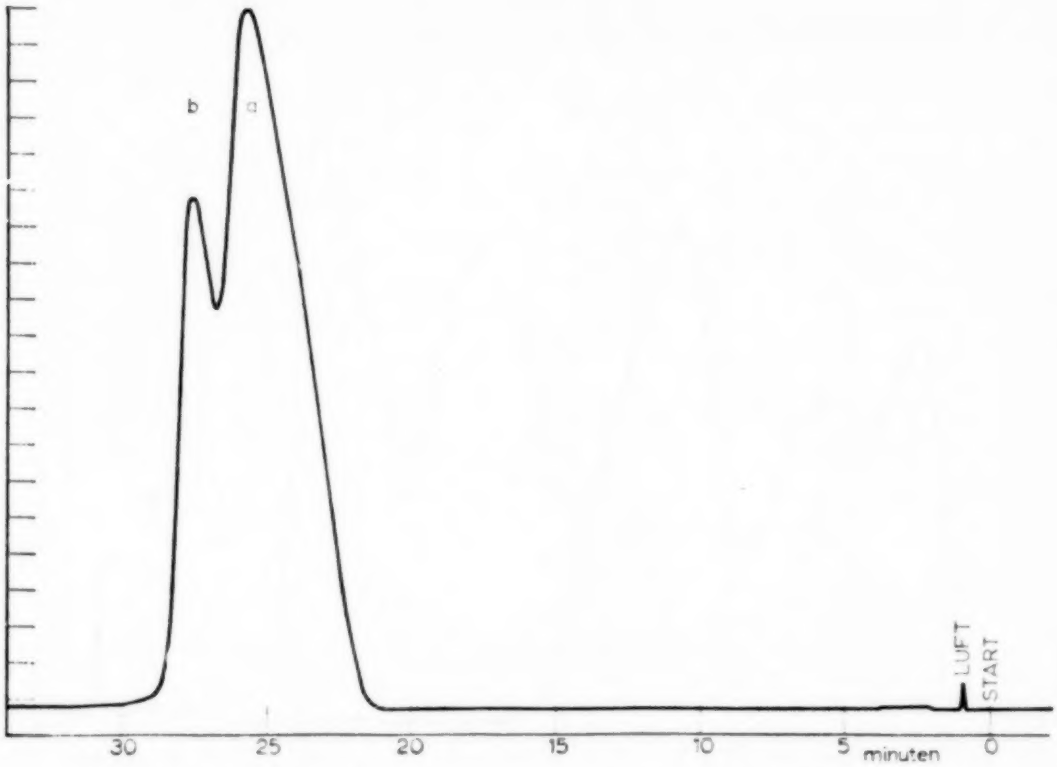

Fig. 1. (a) Neomenthol; (b) Menthol. Stationäre Phase: Siliconöl (Säule C), 2 m; Arbeitstemperatur 180°; Trägergas Helium; Strömungsgeschwindigkeit 40 ml/min.

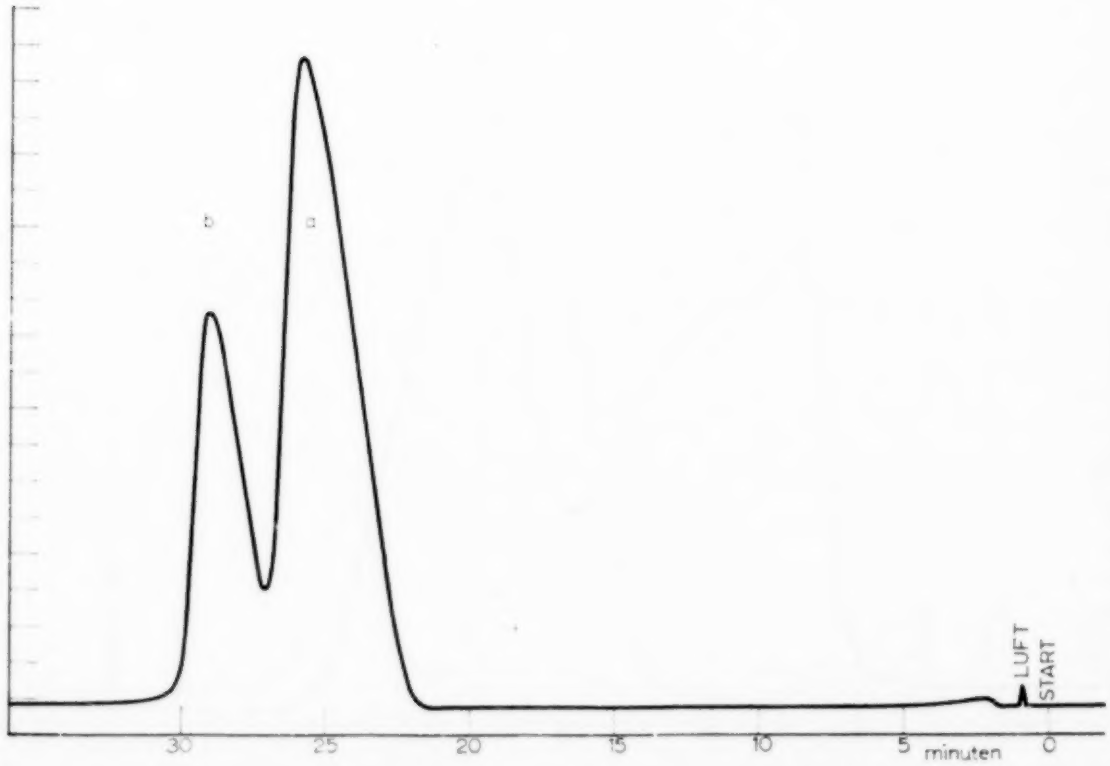

Fig. 2. (a) Neomenthol; (b) Isomenthol. Stationäre Phase: Siliconöl (Säule C), 2 m; Arbeitstemperatur 180°; Trägergas Helium; Strömungsgeschwindigkeit 40 ml/min.

Qualitative Aussagen über die Zusammensetzung von Mentholgemischen erreicht man bereits mit einer A-Säule von 2 m Länge. So erhält man nach Fig. 1 eine Trennung des Menthols vom Neomenthol und nach Fig. 2 eine Trennung des Neomenthols vom Isomenthol (siehe auch Tabelle I, Spalte 1). Hat man jedoch ein Gemisch der drei

Komponenten Menthol–Neomenthol–Isomenthol, so ist es erforderlich, die Säulenlänge auf 4 m zu erhöhen (Fig. 3). Unter diesen Bedingungen erzielt man aber keine eindeutige Trennung des Gemisches Neomenthol–Neoisomenthol–Isomenthol. Erst nachdem die Säulentemperatur auf 160° herabgesetzt worden ist, kann man die drei Substanzen erkennen (Fig. 4, Tabelle I, Spalte 3). Überraschenderweise fallen trotz der bestehenden Dampfdruckdifferenzen die Retentionszeiten von Menthol und Neoisomenthol zusammen, sodass bei gleichzeitiger Anwesenheit in der Mischung

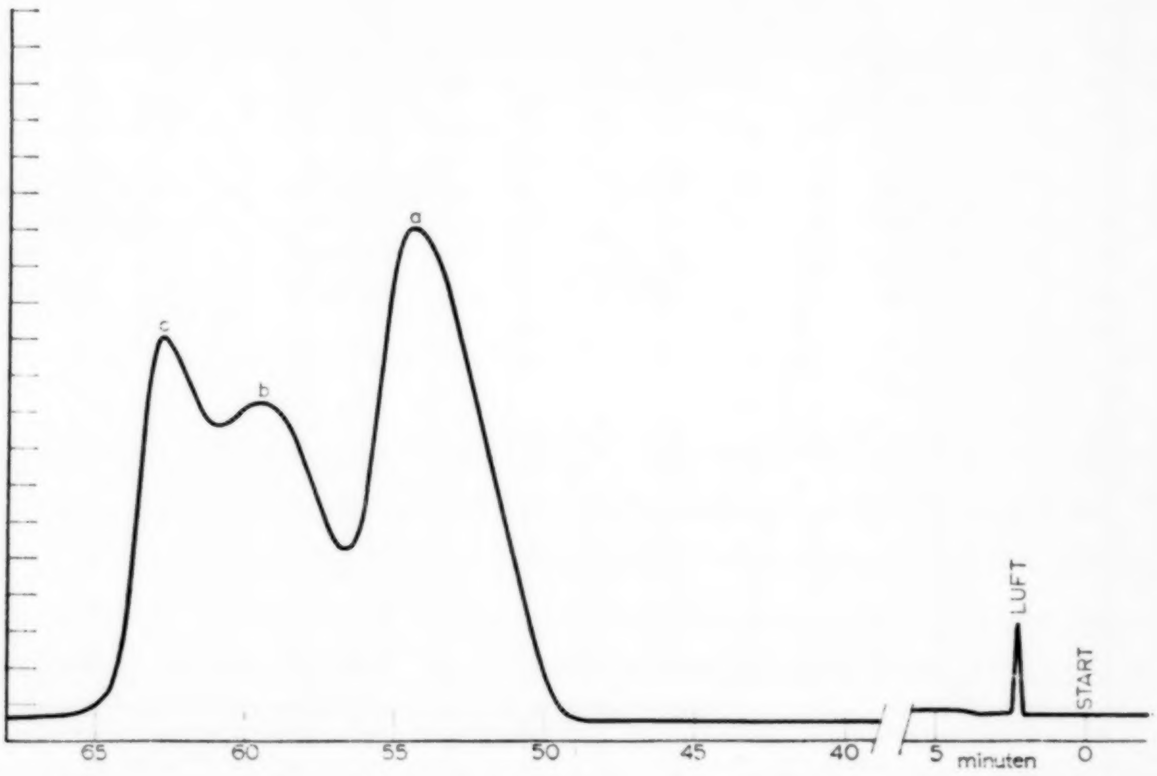

Fig. 3. (a) Neomenthol; (b) Menthol; (c) Isomenthol. Stationäre Phase: Silikonöl (Säule C), 4 m; Arbeitstemperatur 180°; Trägergas Helium; Strömungsgeschwindigkeit 40 ml/min.

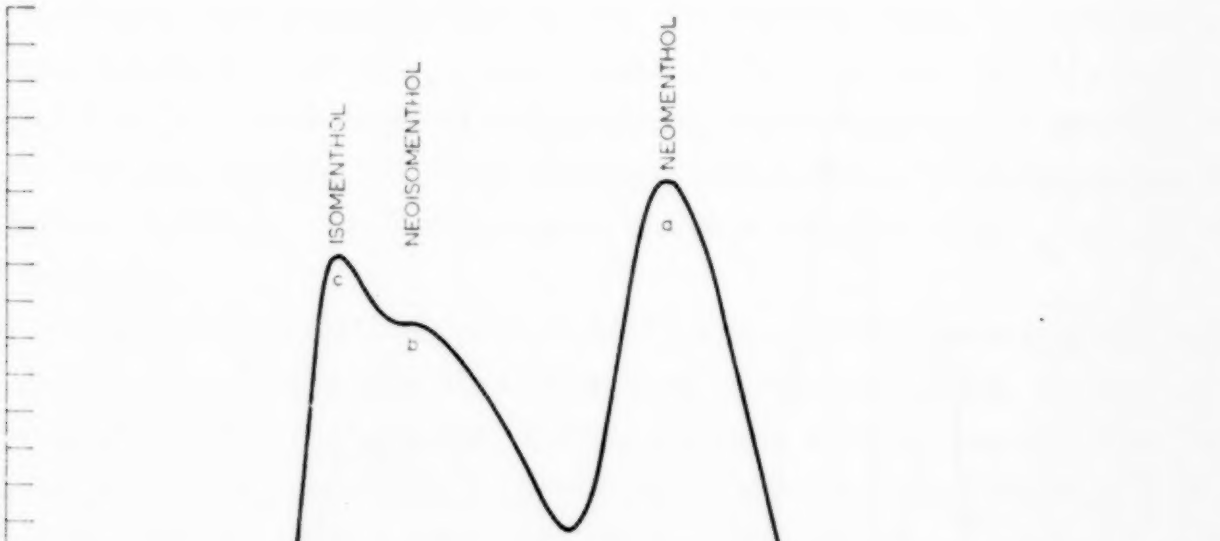

Fig. 4. (a) Neomenthol; (b) Neoisomenthol; (c) Isomenthol. Stationäre Phase: Silikonöl (Säule C), 4 m; Arbeitstemperatur 160°; Trägergas Helium; Strömungsgeschwindigkeit 45 ml/min.

diese beiden Stoffe nicht aufgetrennt werden können. Die Retentionszeiten aus den Chromatogrammen der Einzelkomponenten in Aceton-Lösung (eine solche Auflösung war erforderlich, da Menthol und Isomenthol kristalline Verbindungen sind) weichen von denen ihrer Gemische geringfügig ab und zwar nach niedrigeren Zeiten (Der Neomentholgehalt der Mischungen wurde so hoch gewählt, dass diese flüssig sind.) Ausserdem wurde im Verlaufe der Untersuchungen eine merkliche Beanspruchung der stationären Phase beobachtet, die sich in einer Verkürzung der

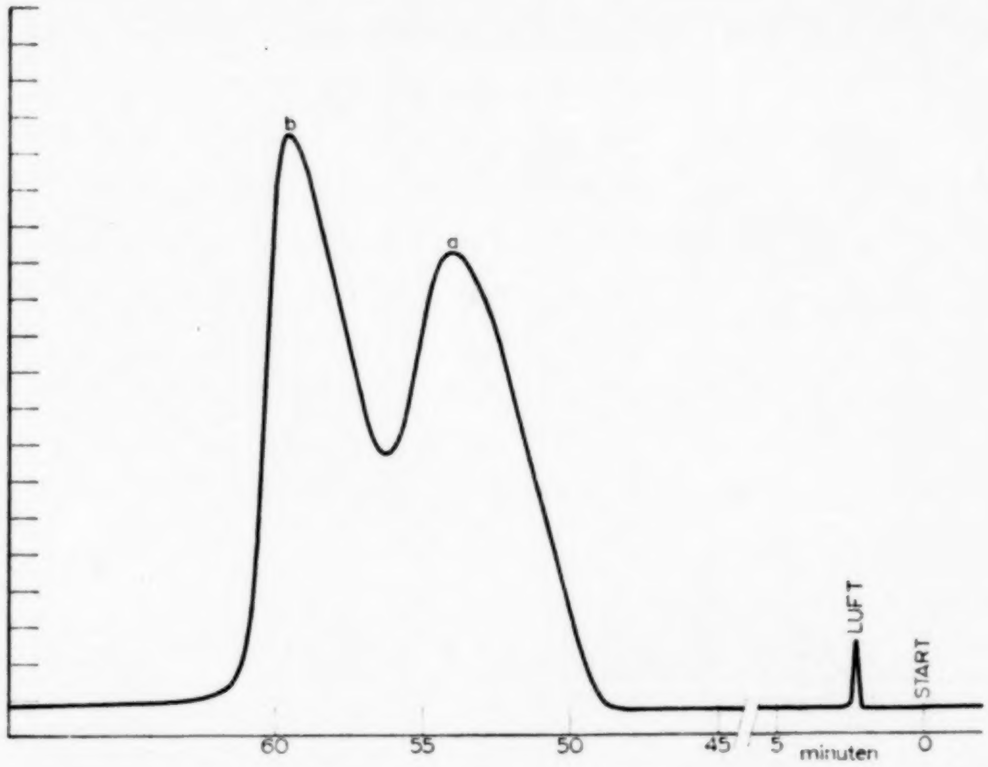

Fig. 5. (a) Neomenthol; (b) Menthol. Stationäre Phase: Siliconöl (Säule C), 4 m; Arbeitstemperatur 180°; Trägergas Helium; Strömungsgeschwindigkeit 40 ml/min.

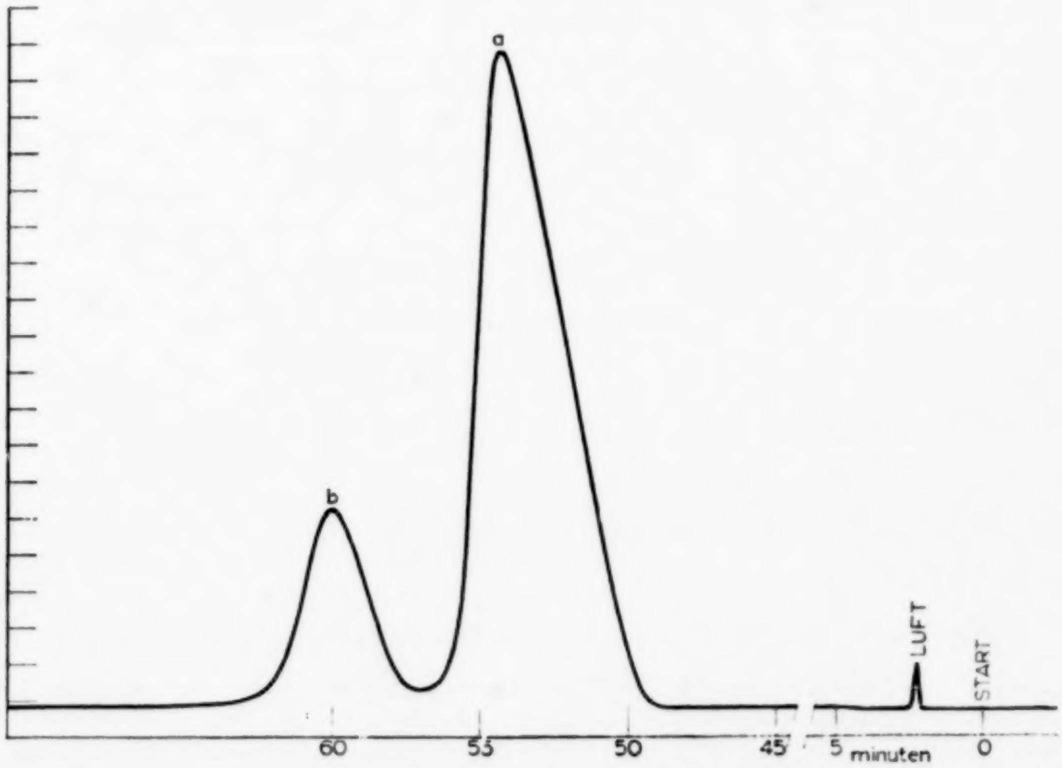

Fig. 6. (a) Neomenthol; (b) Isomenthol. Stationäre Phase: Siliconöl (Säule C), 4 m; Arbeitstemperatur 180°; Trägergas Helium; Strömungsgeschwindigkeit 40 ml/min.

Retentionszeiten zu erkennen gab. Dieser Effekt hat auf die Trennwirkung kaum einen Einfluss. Nach längerer Benutzung einer Säule ist es bei unbekannten Mentholgemischen aber zweckmässig, eine Testanalyse mit einem reinen Isomeren vorzunehmen. Durch die Festlegung der Lage des Test-Peak kann man die einwandfreie Zuordnung der übrigen Isomeren vornehmen, zumal die stereoisomeren Menthole im Chromatogramm in der Reihenfolge ihrer Dampfdrucke erscheinen. Tabelle I enthält die Retentionszeiten (Maximum) bei verschiedenen Bedingungen.

Um die Methode auf ihre Brauchbarkeit zur quantitativen Bestimmung von Mentholgemischen zu prüfen, wurden eine Anzahl Chromatogramme von Gemischen bekannter Zusammensetzung ausgewertet. Zur Ermittlung der prozentualen Zusammensetzung wurden die Flächen der Peaks nach zwei Verfahren bestimmt:

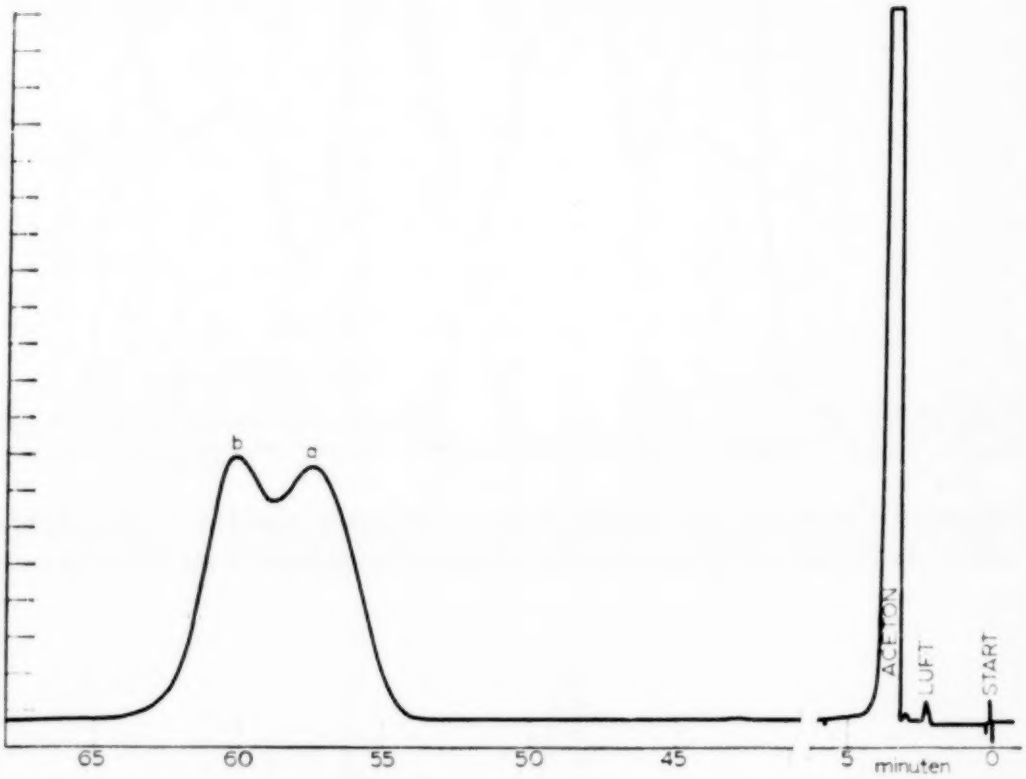

Fig. 7. (a) Menthol; (b) Isomenthol; Gemisch in Aceton gelöst. Stationäre Phase: Siliconöl (Säule C), 4 m; Arbeitstemperatur 180°; Trägergas Helium; Strömungsgeschwindigkeit 40 ml/min.

(a) Wägung der Peaks, die aus dem Chromatogramm ausgeschnitten worden sind[13].

(b) Berechnung nach der Näherungsformel: Peakhöhe × Halbwertsbreite nach Cremer und Müller[14].

In den Tabellen II, III und IV sind die Ergebnisse zusammengefasst. Soweit es sich um kristalline Gemische handelte, wurden ihre Aceton-Lösungen untersucht. Es konnte gezeigt werden (Tabelle II, vgl. Fig. 5), dass ein Gemisch aus Menthol–Neomenthol bereits im Verhältnis 80:20 übereinstimmend nach beiden Methoden mit einer Genauigkeit von ± 1% quantitativ bestimmt werden kann. Die quantitative Zusammensetzung von Neomenthol–Isomenthol-Gemischen kann in jedem beliebigen Mischungsverhältnis aus ihren Chromatogrammen (z.B. Fig. 6) errechnet werden (Tabelle III). Eine quantitative Auswertung der Chromatogramme von Menthol–Isomenthol-Gemischen (Fig. 7) ist nur in begrenztem Umfang nach der Methode (b) möglich (Tabelle IV).

TABELLE II

| *Verhältnis Menthol/Neomenthol* | *Analysenergebnis* | |
|---|---|---|
| | *nach Verfahren (a)* | *nach Verfahren (b)* |
| 90/10 | nicht auswertbar | nicht auswertbar |
| 80/20 | 79.1/20.8 | 79.5/20.3 |
| 70/30 | 70.6/29.3 | 70.1/30.7 |
| 60/40 | 59.2/40.8 | 59.5/40.5 |
| 50/50 | 51.1/48.8 | 51.3/48.6 |
| 40/60 | 39.6/60.3 | 39.5/60.5 |
| 30/70 | 30.9/69.0 | 30.5/69.5 |
| 20/80 | 20.5/79.4 | 21.0/79.0 |
| 10/90 | 10.5/89.5 | 10.2/89.8 |

TABELLE III

| *Verhältnis Neomenthol/Isomenthol* | *Analysenergebnis* | |
|---|---|---|
| | *nach Verfahren (a)* | *nach Verfahren (b)* |
| 90/10 | 89.1/10.9 | 89.9/10.4 |
| 80/20 | 80.8/19.2 | 81.7/18.2 |
| 70/30 | 70.6/29.3 | 70.4/29.7 |
| 60/40 | 61.6/38.3 | 60.6/39.4 |
| 50/50 | 50.9/49.0 | 51.2/48.8 |
| 40/60 | 41.0/59.0 | 41.2/58.8 |
| 30/70 | 30.0/70.0 | 29.8/70.1 |
| 20/80 | 19.5/80.4 | 20.0/80.0 |
| 10/90 | 8.9/91.1 | 9.3/90.7 |

TABELLE IV

| *Verhältnis Menthol/Isomenthol* | *Analysenergebnis* | |
|---|---|---|
| | *nach Verfahren (a)* | *nach Verfahren (b)* |
| 90/10 | nicht auswertbar | nicht auswertbar |
| 80/20 | nicht auswertbar | nicht auswertbar |
| 70/30 | nicht auswertbar | 69.4/30.5 |
| 60/40 | nicht auswertbar | 59.9/40.1 |
| 50/50 | nicht auswertbar | 49.3/50.6 |
| 40/60 | nicht auswertbar | nicht auswertbar |
| 30/70 | nicht auswertbar | nicht auswertbar |
| 20/80 | nicht auswertbar | nicht auswertbar |
| 10/90 | nicht auswertbar | nicht auswertbar |

Die für die Versuche verwendeten stereoisomeren Menthole enthielten keine Verunreinigungen, wie auch aus ihren Chromatogrammen ersichtlich war. Im übrigen hatten sie folgende Konstanten: *d*-Neomenthol, $D_4^{20}$ 0.8992, $n_D^{20}$ 1.4617, $[\alpha]_D^{20}$ + 17.4°; *l*-Menthol, F. 42°; *d*-Isomenthol, F. 82°. Das Neoisomenthol wurde nach HÜCKEL u. Mitarb.[4] durch $LiAlH_4$-Reduktion bei + 20° von Isomenthon $[\alpha]_D^{20}$ + 85° in 90%-iger Ausbeute dargestellt. Nach der Drehbanddestillation (K.p.$_5$ 84°) hatte es folgende Konstanten: $D_4^{20}$ 0.9098, $n_D^{20}$ 1.4668, $[\alpha]_D$ + 0.1°.

## ZUSAMMENFASSUNG

Die Analyse stereoisomerer Menthole in ihren Mischungen durch Gasverteilungschromatographie gelingt bei Verwendung von Di-*n*-decylphthalat als stationäre Phase und Helium als Trägergas. In vielen Fällen ist es möglich, die Chromatogramme für quantitative Bestimmungen heranzuziehen.

## SUMMARY

Mixtures of stereoisomeric menthols can be analysed by means of gas partition chromatography, di-*n*-decyl phthalate being used as the stationary phase and helium as carrier gas. In many cases it is possible to use the chromatograms for quantitative determinations.

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# ÜBER DIE ANWENDUNG VON ZELLULOSE-IONENAUSTAUSCHERN UND ALGINSÄURE ZUR CHROMATOGRAPHISCHEN REINIGUNG UND TRENNUNG DER VITAMINE DER $B_{12}$-GRUPPE

J. PAWEŁKIEWICZ, W. WALERYCH
*Biochemisches Institut der Landwirtschaftlichen Hochschule Poznań (Polen)*

UND

W. FRIEDRICH UND K. BERNHAUER
*Biochemisches Forschungslaboratorium der Aschaffenburger Zellstoffwerke A.G., Stockstadt am Main (Deutschland)*

(Eingegangen den 11. Juli 1959)

## EINLEITUNG

Die bisher zur Trennung von Vitamin $B_{12}$-Arten am meisten verwendeten Methoden sind Zellulosepulver-Chromatographie und Elektrophorese. Sie ergänzen einander, wobei die erste vor allem bei der Trennung neutraler bzw. nicht ionisierbarer $B_{12}$-Arten unersetzlich ist und die zweite zur Trennung ionisierbarer, d.h. basischer oder saurer $B_{12}$-Arten besonders gute Dienste leistet.

Die elektrophoretische Methode hat den Nachteil, dass sie nur in relativ kleinem Masstab anwendbar ist und stets eine Verunreinigung der zu trennenden Proben mit Elektrolyten mit sich bringt.

Unter den Ionenaustauschern hat sich unseres Wissens nur der carboxylhaltige Austauscher Amberlite IRC-50 (XE-64) bewährt, und zwar zur Beseitigung von Salzen, Proteinen und anderen Verunreinigungen. Er ermöglicht jedoch keine Trennung der einzelnen $B_{12}$-Arten.

Die Ursache für die beschränkte Verwendbarkeit der Harzaustauscher liegt sowohl in ihrer Beschaffenheit, als auch in der Art des $B_{12}$-Moleküls. Die Harzaustauscher haben im allgemeinen eine komplizierte, vernetzte Struktur, die den Zugang grosser Moleküle zu den aktiven Gruppen beeinflusst. Dadurch werden diese Moleküle in extremen Fällen entweder gar nicht oder irreversibel festgehalten.

Eine chromatographische Methode, die in der Lage wäre, ionisierbare $B_{12}$-Arten ähnlich wie durch Elektrophorese zu trennen, erschien daher sehr wünschenswert.

Vor kurzem wurden Zellulose-Ionenaustauscher zur Trennung von Proteinen, Peptiden und Nucleinsäuren mit gutem Erfolg eingeführt[1,2]. Es erschien daher aussichtsreich zu prüfen, ob solche Austauscher auch auf dem Vitamin $B_{12}$-Gebiet anwendbar sein mögen, umsomehr, als die genannten Austauscher eine vernetzungsfreie Struktur besitzen, die auch grossen Molekülen den Zugang zu den aktiven Gruppen gestatten. Aus dem gleichen Grund erschien die Prüfung von Alginsäure berechtigt (zu deren Struktur siehe[3]). Wie in vorliegender Mitteilung gezeigt wird, bewähren sich tatsächlich verschiedene Ionenaustauscher auf Polysaccharidbasis ausgezeichnet zur chromatographischen Reinigung und Trennung von Vitamin $B_{12}$-Arten.

## EXPERIMENTELLES

### *Austauscher*

Die handelsüblichen, zum Teil als Natriumsalze bzw. Hydrochloride vorliegenden Zellulose-Ionenaustauscher[1], wie z.B. Carboxymethylzellulose (CM), phosphorsäurehaltige Zellulose (P), Diäthylaminoäthyl-Zellulose (DEAE), und mit Triäthanolamin und Epichlorhydrin umgesetzte Zellulose (ECTEOLA) werden vor der Herstellung der Säulen in die freien Säuren (durch Ansäuern mit verdünnter Salzsäure auf pH *ca.* 2) bzw. Basen (durch Zusatz verdünnter Ammoniaklösung bzw. Natriumhydroxyd) überführt. Die anschliessend gründlich mit Wasser geschlämmten und dann mit Aceton gewaschenen Austauscher werden bei 37° getrocknet. Die handelsübliche fein gemahlene Alginsäure* wird vor Gebrauch gründlich mit Wasser gewaschen.

Zur Herstellung von Säulen suspendiert man die Austauscher in dem entsprechenden Entwickler und füllt den erhaltenen Brei ein. Auf ein Stopfen der Säulen kann zumeist verzichtet werden, um ihre Durchlässigkeit nicht zu stark herabzusetzen.

Brauchbare Austauschersäulen kann man auch durch Niederschlagen von Alginsäure an Zellulosepulver herstellen.

12.5 g Zellulosepulver werden in eine Lösung aus 2 g Natriumalginat* in 60 ml Wasser eingerührt, der Brei wird kurz abgesaugt und der Filterrückstand im Vakuum getrocknet. 5 g des Trockenpulvers werden in 50 ml 0.05 *N* HCl suspendiert, die gut homogenisierte Suspension in das Chromatographierrohr gefüllt und durch Waschen mit Wasser von anhaftender Salzsäure befreit.

Die Säulen sind gut durchlässig und die Alginsäure haftet fest an den Zellulosepartikeln. Linterspulver gibt durchlässigere Säulen als Holzzellulosepulver. Beim Regenerieren mit alkalischen wässrigen Lösungen wird das Alginat ausgewaschen, wonach die Zellulose frisch beladen werden muss.

### *Elutionstechnik*

Die zu trennenden bzw. zu reinigenden Proben werden als wässrige, wässrig alkoholische oder alkoholische Lösungen aufgetragen. Es wird meist rasch (durchschnittlich während einiger Stunden) entwickelt, um ein Verwischen der Zonen durch Diffusion möglichst zu vermeiden.

Man kann zumeist mit elektrolytfreien Flüssigkeiten, also z.B. mit reinem Wasser entwickeln. Stark adsorbierte Stoffe können mit wässrigen Lösungen von wechselnder Konzentration an $H^+$-Ionen bzw. Salzen eluiert werden, wobei die aus der Chromatographie von Proteinen bekannten Grundsätze gelten[2]. Danach wird die Elution durch folgende Änderungen am Entwicklersystem gefördert: (a) Erhöhung der Salzkonzentration, (b) Erhöhung des pH-Wertes (bei Kationenaustauschern), (c) Senkung des pH-Wertes (bei Anionenaustauschern). Der Mechanismus der Elution beruht bei (a) auf der Schwächung elektrostatischer Bindungen zwischen dem Austauscher und der adsorbierten Substanz, bei (b) und (c) auf einer Ladungsänderung, sowohl am Austauscher als auch an den adsorbierten Molekülen. Bei der Elution gehen meist kleine Mengen von Stoffen aus den Austauschern in Lösung. Zu deren Beseitigung empfiehlt sich, den Eindampfrückstand der Eluate in Methanol oder wässrigem Aceton aufzunehmen und über Aluminiumoxyd bzw. Kieselgur zu filtrieren.

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* Hersteller: AS Protan, Drammen, Norwegen.

## ERGEBNISSE

*P-Zellulose* (Fa. Serva Entwicklungslabor, Heidelberg) eignet sich ausgezeichnet zur Trennung von Vitamin $B_{12}$-Arten unterschiedlicher Basizität, also z.B. von Vitamin $B_{12}$, Pseudovitamin $B_{12}$, Faktor A und Vitamin $B_{12b}$. Sehr charakteristisch für diesen Zellulose-Austauscher ist die Bildung äusserst scharfer Zonen, vor allem bei Verwendung rein wässriger Medien als Entwickler. Hierzu eignet sich vor allem reines Wasser sowie Pufferlösungen. Die Entwicklung mit reinem Wasser hat den Vorteil, dass salzfreie, evtl. direkt nach dem Einengen kristallisierbare Eluate erhalten werden. Puffer benützt man im allgemeinen nur zur Elution sehr langsamer Zonen (z.B. von Faktor A und Vitamin $B_{12b}$). Man kann aber auch Mischungen aus gleichen Teilen Wasser und Alkoholen mit gutem Erfolg verwenden (dabei sind jedoch die Zonen etwas weniger scharf), wozu Äthanol am besten geeignet ist. Nucleotidfreie Vitamin $B_{12}$-Analoga wie z.B. Faktor B und Faktor $V_{1a}$ werden bei Cyanidmangel an P-Zellulose festgehalten und können durch Zusatz von Blausäure oder Cyanid zum Entwickler als Dicyano-Komplexe eluiert werden. Dieses Verhalten ist auch für CM und für Alginsäure-haltige Austauscher charakteristisch. Mit einer selbst hergestellten P-Zellulose konnten allerdings keine so guten Trennungen erzielt werden wie mit dem genannten Handelsprodukt. P-Zellulose eignet sich auch sehr gut zur Befreiung der Vitamin $B_{12}$-Arten von Proteinen, von denen ein grosser Teil in der Säule festgehalten wird. Als Lösungsmittel eignet sich hierzu sehr gut Methanol bzw. eine Mischung aus gleichen Teilen Wasser und Methanol.

*Alginsäure* lässt sich nur in rein wässrigem Medium anwenden, ein Zusatz von Alkoholen verursacht diffuse Zonen. Sie ähnelt in ihrem Verhalten gegenüber einer Mischung von Vitamin $B_{12}$, Pseudovitamin $B_{12}$ und Faktor A der P-Zellulose. Sie kann regeneriert werden, indem sie nacheinander mit verdünnter NaCl-Lösung und Wasser gewaschen wird.

Mit Alginsäure imprägniertes Zellulosepulver hat in Bezug auf die Trennfähigkeit der $B_{12}$-Arten weitgehend die Eigenschaften der CM-Zellulose.

*CM-Zellulose* (Fa. Serva Entwicklungslabor, Heidelberg), verwendet in Wasser, in wässrigen Pufferlösungen und beschränkt in wässrig-alkoholischen Lösungen, eignet sich gut zur Trennung von Faktor A und Pseudovitamin $B_{12}$ sowie von Faktor A und Vitamin $B_{12}$, schlechter zur Trennung von Vitamin $B_{12}$ und Pseudo-

TABELLE I

TRENNUNG VON VITAMINEN DER $B_{12}$-GRUPPE IN SÄULEN AUS ZELLULOSE-IONENAUSTAUSCHERN UND ALGINSÄURE

| *Austauscher* | *Entwickler* | *Beobachtete R-Werte** | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Fakt. B* | *Fakt. $V_{1a}$* | $B_{12}$ | $\psi$-$B_{12}$ | *Fakt. A* | *Vit. $B_{12}$-carbonsäuren* |
| P-Zellulose | Wasser | 0.0 | 0.0 | 1.2 | 0.15 | 0.02 | 1.2 |
| | Wasser + HCN | 1.2 | 1.2 | | | | |
| | MeOH–$H_2O$ (1:1) | — | — | 1.1 | 0.3 | 0.08 | — |
| | ÄtOH–$H_2O$ (1:1) | — | — | 1.1 | 0.3 | 0.08 | — |
| | IsoprOH–$H_2O$ (1:1) | — | — | 1.0 | 0.23 | s. klein | — |
| | *sec.*-BuOH ges. mit $H_2O$ | — | — | 0.8 | 0.08 | s. klein | — |
| Alginsäure | Wasser | 0.0 | 0.0 | 1.0 | 0.1 | 0.02 | 1.0 |
| | Wasser + HCN | 1.0 | 1.0 | | | | |
| Alginsäure-Zellulose | Wasser | 0.0 | 0.0 | 1.2 | 0.63 | 0.27 | 1.2 |
| | Wasser + HCN | 1.2 | 1.2 | | | | |
| CM-Zellulose | Wasser | 0.0 | 0.0 | 1.0 | 0.8 | 0.33 | 1.0 |
| | Wasser + HCN | 1.0 | 1.0 | | | | |
| | BuOH ges. mit $H_2O$ | — | — | 0.7 | 0.3 | 0.1 | — |

* $R = \frac{\text{Verschiebung der Zone}}{\text{Verschiebung des Entwicklerniveaus}}$

vitamin $B_{12}$. Wohl aber konnten mit Hilfe einer selbst hergestellten CM-Zellulose sehr scharfe Trennungen erzielt werden. Die Eigenschaft von CM-Zellulose, Proteine zu adsorbieren[1, 2], kann zur Reinigung von Vitamin $B_{12}$-Arten benützt werden. Man verwendet dabei wässrige, methanolische sowie auch wässrig-methanolische Medien, wobei ein Teil der Proteine in der Austauschersäule zurückbleibt.

*DEAE-Zellulose* (Fa. Schleicher & Schüll, Dassel) trennt in wässrigen, wässrig-methanolischen sowie in rein methanolischen Lösungen saure $B_{12}$-Arten von neutralen und basischen, indem die sauren zurückgehalten werden und die übrigen die Säule durchlaufen. In Gegenwart eines Überschusses an Blausäure kann DEAE-Zellulose auch zur Trennung von Faktor B von allen anderen $B_{12}$-Arten dienen, da diese unter diesen Bedingungen als saure Dicyanokomplexe vorliegen und daher festgehalten werden. Dieser Austauscher hat eine besonders hohe Kapazität gegenüber Proteinen und Peptiden und eignet sich deshalb ausgezeichnet zur Reinigung von Konzentraten der Vitamin $B_{12}$-Arten. Als Medium wird in diesem Falle meist Methanol oder ein Gemisch aus gleichen Teilen Wasser und Methanol verwendet.

*ECTEOLA-Zellulose* (Fa. Schleicher & Schüll, Dassel) unterscheidet sich in Bezug auf die Trennbarkeit von $B_{12}$-Arten wenig von DEAE. Da sie jedoch eine geringere Kapazität gegenüber Proteinen besitzt[1], wird meist DEAE zu bevorzugen sein.

*TEAE-Zellulose* (Fa. Serva, Entwicklungslabor, Heidelberg) lässt sich, ähnlich wie DEAE-Zellulose, zur Trennung von sauren Vitamin $B_{12}$-Faktoren verwenden. Besonders gut gelingt die Trennung von Vitamin $B_{12}$-Mono-, Di- und Tricarbonsäuren unter Verwendung steigender Mengen an Tris-Puffer*.

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* Nach Versuchen von Frl. Dipl. chem. GISELA GROSS.

TABELLE II

TRENNUNGSGANG VON $B_{12}$-ARTEN DURCH ZELLULOSE-IONENAUSTAUSCHER

| *Kationenaustauscher (P-Zellulose oder CM-Zellulose)* | | | | |
|---|---|---|---|---|
| CN′-armer Entwickler: Durchlauf von neutralen und sauren Stoffen | | CN′-reicher Entwickler: Durchlauf von Dicyanoformen | | Trennung der basischen Stoffe in der Säule: |
| *Anionenaustauscher (DEAE oder TEAE)* | | | | |
| Durchlauf neutraler Anteile:<br>Vit. $B_{12}$, Faktor III, etc. | Trennung der sauren Stoffe in der Säule:<br>Vit. $B_{12}$-carbonsäuren u. phosphorsäurehaltige inkomplette $B_{12}$-Faktoren | Durchlauf neutraler Stoffe:<br>Faktor B | Trennung der sauren Stoffe in der Säule:<br>Faktoren $V_1$–$V_5$ | Pseudo-Vitamin $B_{12}$, Faktor A |

Tabelle II zeigt einen Trennungsgang von natürlichen Vitamin $B_{12}$-Arten mit Hilfe von Zellulose-Ionenaustauschern.

## ZUSAMMENFASSUNG

Es wird über die Verwendung von einigen Zellulose-Ionenaustauschern sowie von Alginsäure und Alginsäure–Zellulose-Präparaten zur chromatographischen Trennung und Reinigung der Vitamine der $B_{12}$-Gruppe berichtet. Die beschriebene Methode besitzt den Vorteil, dass sie sehr rasch und auch in grossem präparativen Masstab durchführbar ist. Sie ergänzt in sehr wertvoller Weise die Zellulosepulver-Chromatographie und Elektrophorese.

## SUMMARY

A study was made of the application of some cellulose ion exchangers, as well as alginic acid and alginic acid–cellulose preparations, for the chromatographic separation and purification of vitamins of the $B_{12}$-group. The advantages of the method described are that it is very rapid and can also be applied for the preparation of large amounts of these vitamins. The technique is a valuable complement to cellulose-powder chromatography and electrophoresis.

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# OPTICAL RESOLUTION BY MOLECULAR COMPLEXATION CHROMATOGRAPHY

L. H. KLEMM AND DAVID REED

*Department of Chemistry, University of Oregon, Eugene, Oreg. (U.S.A.)*

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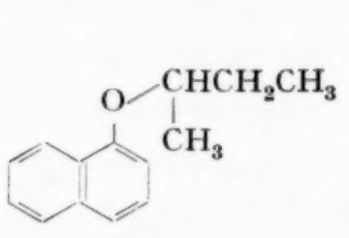
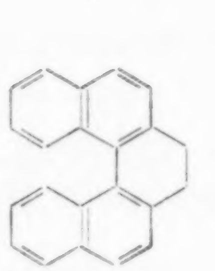
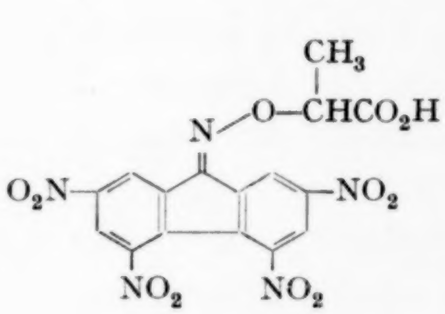
In a previous paper[1] we described the use of columns of silicic acid impregnated with picric acid or 2,4,7-trinitrofluorenone for adsorption chromatography of mixtures of aromatic hydrocarbons. It was proposed that the process of adsorption was identifiable with molecular compound formation between the aromatic hydrocarbon and the polynitro complexing agent held monomolecularly on the surface of the silicic acid. We now report the successful extension of such method to the optical resolution of two racemic compounds, 1-naphthyl 2-butyl ether (I) and 3,4,5,6-dibenzo-9,10-dihydrophenanthrene (II), by use of the optically active complexing agent (+)- or (—)-α-(2,4,5,7-tetranitro-9-fluorenylideneaminooxy)-propionic acid (III) as impregnant.



(I)



(II)



(III)

Synthesis of (III) and its use in optical resolution of (I) and other racemates by means of fractional crystallization or precipitation of molecular complexes from solution has been effected by NEWMAN AND LUTZ[2] and NEWMAN AND LEDNICER[3]. Optically active (II) has been previously obtained only by chemical transformation of resolved 1,1′-binaphthyl-2,2′-dicarboxylic acid[4]. The use of columns of alumina impregnated with various naturally occurring optically active compounds for accomplishing partial resolution of racemates has been described recently by KARAGOUNIS, CHARBONNIER AND FLÖSS[5], who have also presented a bibliography on optical resolution by means of chromatography on pure optically active absorbents.

## EXPERIMENTAL

### *Preparation of impregnated adsorbent*

To a column (4.5 × 41 cm) of Mallinckrodt reagent-grade 100-mesh silicic acid (packed dry in an all-glass tube, shielded from light by means of aluminum foil wrapping, and under the vacuum provided by a water aspirator) was added, in succession, 100 ml of reagent-grade chloroform (prewash), a solution of 31.4 g of (—)-III[2], m.p. 151.5–155°, $[\alpha]_D^{24}$ —82.5° (*c* 0.813, dioxane), in 250 ml of chloroform, 50 ml of chloroform, and 1 l of Mallinckrodt reagent-grade 30–60° petroleum ether. Thereafter, air was sucked through the column for 12 h. Small portions at the top and bottom of the column were dug out and discarded. The main part of the impregnated silicic acid was dried further in a vacuum desiccator for 24 h, pulverized by means of a mortar and pestle, and stored in a brown bottle, yield 354 g of impregnated adsorbent containing 8.9% (by weight) of (—)-III ($2.1 \cdot 10^{-4}$ moles of III per g). Silicic acid impregnated with (+)-III was prepared in the same manner.

### *Chromatography*

Using the suction of a water aspirator a 1.9-cm (diameter) tube was packed dry with successive layers of 1–2 cm of plain silicic acid, 40 cm (75–85 g) of impregnated silicic acid, and 1 cm of purified sand. The column was prewashed with 100–200 ml of petroleum ether. A solution of the racemic or partially resolved compound in the minimum volume of petroleum ether was added to the column (still under suction) and development and elution were conducted with the same solvent. Development of the chromatogram was readily observed by the formation of a dark red to brown color (molecular compound) which gradually expanded downward on the column. As soon as this color reached the bottom of the column, samples of effluent were collected arbitrarily (3–8 fractions) and evaporated to dryness in a stream of nitrogen. Each resultant residue was redissolved in approximately 5 ml of a suitable solvent (petroleum ether for I, benzene for II), transferred to a 2-dm polarimeter tube, diluted with the same solvent to fill the tube, measured polarimetrically, transferred quantitatively to a tared flask, re-evaporated, dried *in vacuo* for 1–2 h, and weighed. By selective recombination and re-chromatography of effluent fractions, samples of (I) and (II) in various degrees of optical purity were obtained; maximum $[\alpha]_D^{24}$ values found, + 7° (*c* 0.143, petroleum ether) for (I), — 1300° (*c* 0.014, benzene) for (II); reported $[\alpha]_D^{29}$ — 7.4° (*c* 2.03, ethyl acetate)[2] for (I), $[\alpha]_{5791}^{22}$ — 1307° (*c* 0.525, benzene)[4] for (II). Recovery of at least 90% of the adsorbate charged to the column was usually accomplished in these runs. Illustrative data are presented in Table I. Also in an exploratory run using (+)-III and racemic methyl α-(1-anthryl) propionate[2] (IV), it was found that (—)-IV was eluted first.

In a test run 18.4 mg of (II), $[\alpha]_D^{23}$ + 584°, was passed through a 0.9 × 23-cm column of plain silicic acid. The total collected effluent contained 16.0 mg of residue, $[\alpha]_D^{24}$ + 574°. Analogous results were obtained with a column of alumina.

### *Optical resolution by crystallization*

Excess hydrocarbon was allowed to crystallize from a solution containing 200 mg of (±)-II and 150 mg (*ca.* 0.5 molar equivalent) of (+)-III in 5 ml of benzene. The crystalline precipitate was collected by filtration, washed with 2 drops of benzene,

TABLE I

SELECTED DATA ON OPTICAL RESOLUTION BY MOLECULAR COMPLEXATION CHROMATOGRAPHY

| Run | Adsorbate used | | | Adsorbate solution *ml* | Optical sign of impregnant | Time of run *h* | Effluent fraction | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *formula* | *mg* | $[\alpha]_D^{24}$ *(degrees)* | | | | *No.** | *ml* | *mg of residue* | *observed*** *α (degrees)* | $[\alpha]_D^{24}$ *(degrees)* |
| A | (I) | 2500 | 0 | 15 | — | 27 | 1 | 40 | 1439 | — 0.06 | — 0.3 |
| | | | | | | | 2 | 100 | 570 | + 0.02 | + 0.2 |
| | | | | | | | 3 | 250 | 378 | + 0.03 | + 0.5 |
| B | (I) | 150 | —2.5 | 5 | — | 23 | 1 | 40 | 41 | — 0.02 | — 3 |
| | | | | | | | 3 | 50 | 34 | + 0.02 | + 4 |
| | | | | | | | 4 | 350 | 18 | + 0.02 | + 7 |
| C | (II) | 1000 | 0 | 2000 | + | 16 | 1 | 250 | 131 | + 0.54 | + 26 |
| | | | | | | | 3 | 450 | 197 | 0.00 | 0 |
| | | | | | | | 6 | 250 | 87 | — 0.64 | — 46 |
| D | (II) | 87 | —46 | 110 | + | 4.5 | 1 | 20 | 2.2 | + 0.08 | + 230 |
| | | | | | | | 2 | 30 | 8.1 | + 0.26 | + 200 |
| | | | | | | | 4 | 100 | 38.9 | — 0.97 | — 150 |
| E | (II) | 131 | +26 | 250 | — | 38.5 | 1 | 50 | 11.1 | — 0.73 | — 410 |
| | | | | | | | 2 | 35 | 13.1 | — 0.35 | — 170 |
| | | | | | | | 6 | 80 | 17.2 | + 0.78 | + 280 |
| | | | | | | | 7 | 100 | 7.4 | + 0.40 | + 340 |
| F | (II) | 37.0 | —540 | 15 | — | 56.1 | 1 | 50 | 1.2 | — 0.22 | — 1100 |
| | | | | | | | 2 | 50 | 3.0 | — 0.55 | — 1100 |
| | | | | | | | 3 | 50 | 3.5 | — 0.59 | — 1000 |
| | | | | | | | 4 | 75 | 5.3 | — 0.71 | — 830 |
| | | | | | | | 7 | 300 | 5.5 | 0.00 | 0 |
| G | (II) | 12.8 | —990 | 10 | — | 25.5 | 1 | 200 | 1.8 | — 0.38 | — 1300 |
| | | | | | | | 2 | 200 | 6.2 | — 1.16 | — 1200 |
| | | | | | | | 3 | 450 | 3.2 | — 0.37 | — 720 |

* Fractions are numbered consecutively in order of appearance.

** For a solution of the total residue.

and dissolved in 10 ml of benzene. The benzene solutions were washed with excess saturated sodium bicarbonate and measured polarimetrically in the aforementioned manner. From the precipitated fraction was obtained 20.1 mg of II, $[\alpha]_D^{24}$ + 568°; from the filtrate, 172.7 mg of II, $[\alpha]_D^{24}$ — 64.1°.

## DISCUSSION

During each chromatographic run with impregnated silicic acid (*cf.* Table I) the effluent was observed either to change sign of optical rotation or (where such change of sign did not occur) to decrease in absolute magnitude. It is thus clear that optical resolution was effected by the column. However, observed polarimeter readings were too small in the case of (I) to ascertain accurately the optical purities of the samples. For (II), on the other hand, optical purity of at least 80% would appear to have been achieved in some samples. There seems to be little reason to doubt that extension of our procedure could give considerably larger samples of complete optical purity.

The optical sign of the impregnant determines which enantiomorph is retained more selectively on the column. Thus for (II), when (+)-III was used on the column (—)-II was more strongly retained; while when (—)-III was used, (+)-II was more strongly retained. In all runs with (I), where only (—)-III was used, excess (—)-enantiomorph appeared in the first portions of effluent. Combined with the fact that silicic acid by itself causes neither further optical resolution nor racemization of partially resolved (II), these data confirm the presumption that it is the optically active impregnant (and not the silicic acid) which is responsible for the observed optical resolution. Moreover, the intense colors produced on the columns plus the chemical natures of (I)–(IV) are strong indications that this resolution is effected through *bona fide* molecular compound formation on the surface of the silicic acid, from which (III) is not removed by washing with petroleum ether. It was pointed out previously[1] that adsorbability on impregnated silicic acid is independent of relative values of melting point for the adsorbate components or the molecular compounds formed. It has also been postulated[6] that neither relative melting point nor relative solubility is pertinent to adsorption on alumina. Likewise, in the present studies the enantiomorphs are separated despite the fact that their melting points and solubilities would be identical. Moreover, since the complexing agent does not move on the column (*i.e.* does not enter the mobile phase), the relative solubilities of the molecular compounds formed *in situ* cannot be pertinent to the chromatographic process. Thus in glacial acetic acid (—)-III·(+)-I is more soluble than (—)-III·(—)-I, and (+)-III·(—)-IV is more soluble than (+)-III·(+)-IV[2]. Though comparison is confused by the fact that different solvents were used in the resolution by chromatography and by crystallization, in the case of I the enantiomorph which forms the less soluble complex is eluted first and in the case of IV that which forms the less soluble complex is eluted last.

Examination of Table I (runs C, E and F) indicates that the highest efficiency of resolution is gained by using small weights of adsorbate (and/or small volumes of adsorbate solution) and slow flow rates through the column. These are conditions conducive to fractionation *via* virtual equilibrium processes such as

$$(+)\text{-III}_{(\text{adsorbed})} + (+)\text{-II}_{(\text{solution})} \rightleftharpoons [(+)\text{-III}\cdot(+)\text{-II}]_{(\text{adsorbed})}$$

and

$$(+)\text{-III}_{(\text{adsorbed})} + (-)\text{-II}_{(\text{solution})} \rightleftharpoons [(+)\text{-III}\cdot(-)\text{-II}]_{(\text{adsorbed})}$$

where relative adsorbabilities of the enantiomorphs are determined fundamentally by the relative thermodynamic stabilities of their adsorbed molecular compounds. Since our total column contains $1.7 \cdot 10^{-2}$ moles of impregnant and the percentage of these molecules complexed at any one time may be small, it follows that our method can scarcely be expected to compete with optical resolution by fractional precipitation methods on a macro-preparative scale. On the other hand our procedure would appear to be feasible for (a) the resolution of relatively small amounts of material, (b) the determination of relative stabilities of the diastereoisomeric molecular compounds, and (c) possibly, in the last analysis, assignment of absolute configuration(s) to the impregnant or the adsorbate enantiomorphs used.

## ACKNOWLEDGEMENTS

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## SUMMARY

Partial optical resolutions of three racemic aromatic compounds have been achieved by chromatography on silicic acid impregnated with optically active α-(2,4,5,7-tetranitro-9-fluorenylideneaminooxy)-propionic acid using petroleum ether as solvent. The success of this method is ascribed to the occurrence of molecular complexation (between the widely dispersed molecules of the impregnant and the aromatic compound) on the surface of the silicic acid support.

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# PAPER ELECTROPHORESIS OF PROTEINS IN ACID BUFFER

JERRY H. GELLER, JONATHAN H. CUSTER AND CHARLES A. ZITTLE
*Eastern Regional Research Laboratory**, *Philadelphia, Pa.* (*U.S.A.*)

(Received August 5th, 1959)

Paper electrophoresis of proteins has been generally limited to pH values alkaline to the isoelectric points of proteins because positively charged proteins become adsorbed to paper[1]. However, some mixtures of protein are more separable by conventional electrophoresis in acid media than in alkaline media; such is the case for $\alpha$-casein[2]. Although $\alpha$-casein appears as a single component at pH 8.6 (veronal buffer), it exhibits two or more components at pH 2.3 using free-boundary electrophoresis[2]. These components differ in their sensitivity to precipitation by calcium ions. They have been referred to descriptively as calcium-sensitive $\alpha$-casein and calcium-insensitive $\alpha$-casein. Since paper electrophoresis has advantages over free-boundary electrophoresis for fractionation work, a suitable technique in the pH 2.3 region on paper was considered desirable.

Buffer materials for the pH 2.3 region are limited since many organic acids do not ionize sufficiently to provide buffering capacity there. Glycine, although adequate in this respect, produces turbidity in some casein solutions. Hydrochloric acid cannot be used in the Durrum type cell for paper electrophoresis because free chlorine is liberated at the anode. In addition to these limitations for acid electrophoresis of proteins, the limitation caused by the tendency of $\alpha$-casein to travel as irregular broad bands at pH 8.6 (see Fig. 1) is also encountered in acid solution unless special steps are taken. In this study, these steps have been determined; applied to the $\alpha$-casein complex and to its components, they permit the paper electrophoretic separation of bands corresponding to calcium-sensitive and calcium-insensitive $\alpha$-caseins.

Paper electrophoresis was carried out in a Spinco Model R (Durrum type) cell. In each run, 8 strips of S & S 2043A filter paper were used; each strip measured 30.6 × 3.6 cm. The casein electropherograms were stained in accordance with procedures reported by LEVITON[4] for milk proteins.

The effect of various buffer materials on the adsorption of positively charged $\alpha$-casein to filter paper was determined by a simple chromatographic procedure. If a spot of 1% casein was placed on a filter paper strip and chromatographed with an aqueous solution of buffer material, it could be seen that the spot (1) did not move with the solvent (adsorption), (2) moved as a streak from the origin to the solvent front (partial adsorption), or (3) moved as a spot with the solvent front (no adsorption, and therefore ideal buffer material). When it was found that certain additives enhanced the buffer qualities, these materials were also used as chromatographic solvents even though they could not serve as buffer materials alone.

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* Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

The following materials were screened by this chromatographic technique; glycolic acid; oxalic acid; lactic acid; acetic acid; propionic acid; galactose, sucrose, sodium potassium tartrate[5]; sodium octyl sulfate. Lactic acid was the only material which permitted minimal adsorption of the α-casein complex (one component was adsorbed) and provided adequate buffering at pH 2.3. When propionic acid was added to the lactic acid, no components of α-casein were adsorbed to the paper. The other materials tested were unsuitable. Several other α-hydroxyl acids besides lactic acid have sufficient dissociation values to permit buffering at pH 2.3 (α-hydroxybutyric, -valeric, and -caproic) but their expense or unpleasant odor will probably limit their use.

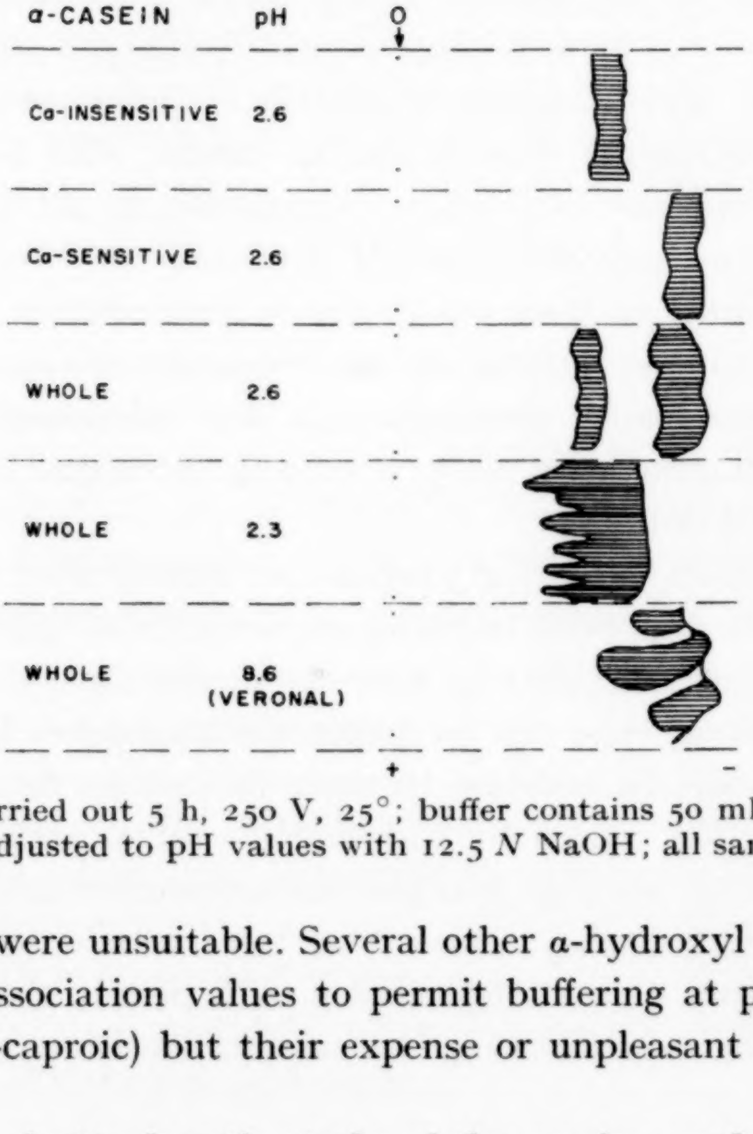

Fig. 1. Electrophoresis carried out 5 h, 250 V, 25°; buffer contains 50 ml lactic acid and 20 ml propionic acid per l, adjusted to pH values with 12.5 $N$ NaOH; all samples are α-casein.

The results of the electrophoretic study of the caseins confirmed the results of chromatography; that is, a material conducive to a smeared chromatographic strip was also conducive to a smeared or blurred electrophoretic strip. The electrophoretic patterns using lactic acid alone or lactic acid plus propionic acid exhibited the same adsorption characteristics as the chromatographic patterns produced by them.

Even the buffer mixture (50 ml lactic acid (85% purity) and 20 ml of propionic acid per liter, adjusted to pH 2.3) did not provide ideal paper electrophoretic patterns of the α-casein complex at 4°, since a densely stained component still moved as several broad streaks rather than a sharp band (Fig. 1). Electrophoresis at several pH values and several temperatures indicated that sharp bands are produced at pH 2.6 and 25°.

When 8 strips were used in the Durrum cell run at room temperature (25°), it was found that a 5 to 10 hour run at 250 V and 8 mA average was ideal. Overnight runs (16–17 h) at lower voltages (200 V) permitted too much diffusion to take place. Amperages as high as 16 mA caused such rapid evaporation that the apex of the paper strips became dry and further protein migration was halted. When the propionic acid content was increased from 20 ml to 22 ml/l, the bands produced were not as sharp as formerly.

Placing the samples at positions removed from the apex of the strips was of no advantage. The buffer flow—caused by evaporation from the strips and consequent replacement by reservoir buffer—was studied by placing lactose samples at various positions. It appeared that when electrophoretic migration was in the same direction as buffer flow, the bands were further apart, but more diffuse. When buffer flow was in the opposite direction, the bands were closer and sharper.

The slight "streaming" effect in the patterns did not alter the band positions. If this effect was produced by evaporation as suggested by SLOTTA *et al.*[6], it could be eliminated by horizontal electrophoresis between glass plates.

Samples of the $\alpha$-casein complex, calcium-sensitive and calcium-insensitive $\alpha$-caseins could be distinguished on paper electrophoresis with the conditions developed. These conditions consisted of the use of 50 ml of lactic acid, 20 ml of propionic acid, and 2 ml of 12.5 *N* NaOH per l giving pH 2.6 buffer and the running of samples in a Durrum cell with 8 strips for 5 h at 250 V, 8 mA (average) at 25°.

The $\alpha$-casein complex exhibited bands at 4.0 cm and 6.0 cm. Calcium-insensitive $\alpha$-casein produced one band at 4.3 cm and calcium-sensitive $\alpha$-casein produced one band at 6.0 cm. The bands are illustrated in Fig. 1 along with patterns produced at pH 8.6 and pH 2.3 to demonstrate the improvement in resolution at pH 2.6

## SUMMARY

An acid buffer solution (pH 2.3 to 2.6) has been devised, which permits paper electrophoresis of $\alpha$-casein complex and its components. This buffer consists of lactic acid and propionic acid; it minimizes adsorption of protein to filter paper and produces sharp bands, which are characteristic of calcium-sensitive and calcium-insensitive $\alpha$-caseins. Electrophoresis is carried out at 25° at 250 V for 5 hours.

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## Short Communications

### Ionization detector for gas chromatography: A modification without radiation source

LOVELOCK's detector[1] can be used without radiation source if it is constructed of glass. This modification is especially useful for the analysis of methyl esters of fatty acids. It seems to possess the same characteristics of response as the $^{90}$Sr detector. The absence of the strontium plate causes a considerable fall of the basic ionization current and increases the stability of the detector at higher anode potentials and gives a lower noise level. The sensitivity is at least of the same order as that of LOVELOCK's detector.

The original problem was to find out whether the radioactive samples emerging from the column could replace the $^{90}$Sr source. It was expected that only radioactive fractions would be indicated, when the $^{90}$Sr source was removed. However, all the fractions gave a response.

To facilitate the cleaning of the ionization chamber, the detector was made of two parts, connected by a B-29 ground Pyrex glass joint (Fig. 1). The central electrode was a 12 × 0.5 mm platinum disk. Either a radiation source of 10 mC $^{90}$Sr in a 0.3 × 25 × 71 mm silver plate (Code SIC 9, Radiochemical Centre, Amersham, Bucks.) or an aluminium plate served as anode. The electrical connections to both electrodes were of platinum wire.

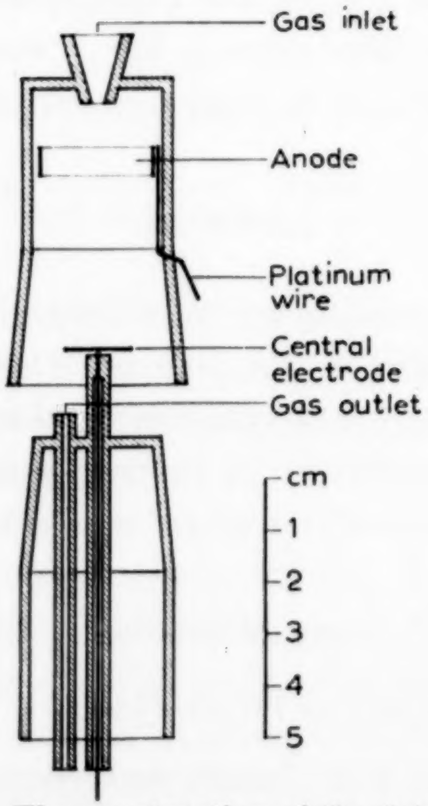

Fig. 1. The construction of the detector.

about 6 mm
plate-to-ring
spacing, asse
Operates in
region of
incipient coro
(1000-1600 V)

The response of this detector was studied in a gas chromatography apparatus according to JAMES[2], which was made in our laboratory using a 4 ft. glass column of 4 mm diameter filled with Celite 545 of 100–120 mesh containing 20 % (w/w) Apiezon L grease as stationary phase. Commercial argon (99.8 %) at an inlet pressure of 0.5 atm. was used as carrier gas. The column and the detector were maintained at a temperature of +180°. The theoretical plate number was about 1500 (calculated for methyl laurate). The ionization current was amplified by a feedback electrometer amplifier with 5000 M$\Omega$ input resistance and recorded by a 50 mV Honeywell recorder.

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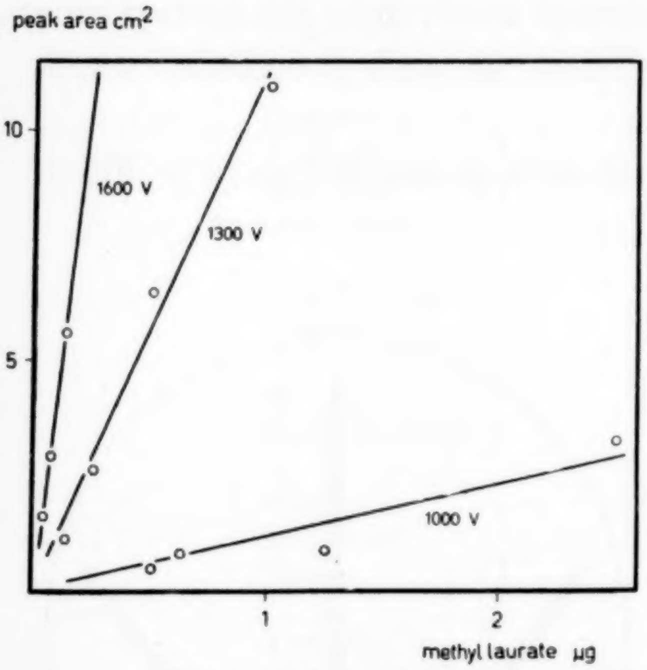

Fig. 2. The recorded peak area, when various amounts of methyl laurate were introduced into the detector. The lines obtained with three different anode potentials (1000, 1300 and 1600 V) are shown.

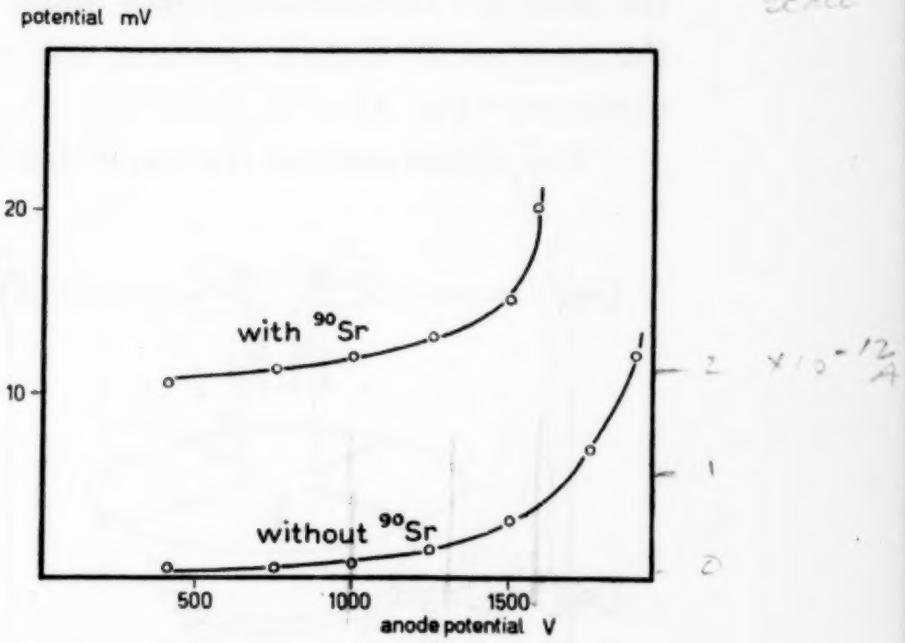

Fig. 3. The central electrode potential in pure argon with a $^{90}$Sr source or with an Al-plate as the anode.

Fig. 2 illustrates the response of the detector, as indicated by the recorded peak areas plotted against the amount of methyl laurate ($\mu$g) introduced into the column. Using a potential of 2000 V and applying 0.1 $\mu$l of a 1:200,000 dilution, the detector still gave a well detectable response (peak area 0.36 cm$^2$) which corresponds to $5 \cdot 10^{-4}$ $\mu$g of methyl laurate. The quality of the records was not affected. In Fig. 3 the central electrode potential is plotted against the voltage applied to the anode, with pure argon in the detector.

We suggest that the responses are due to differences in the conductivity of the gases passing the detector.

We are greatly indebted to Mr. R. SAARI, Eng. D., who assisted in the design of the amplifier and to Pharmaceutical Manufacturers Lääke Oy, Turku, who gave us financial support.

*Department of Medical Chemistry, University of Turku (Finland)*
E. HAAHTI
T. NIKKARI
E. KULONEN

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# Time saving applications in horizontal paper chromatography of amino acids

The method published previously by the authors[1] separates the mixture of amino acids with the first solvent system into several groups, transfers these onto another paper by capillary elution and effects the separation of the groups into individual amino acids with other solvent systems.

This method has been improved. A solvent system[2] was found which eliminates the need for rechromatography and a simple apparatus which dries the spots during the process of elution permits one worker to control six such procedures simultaneously (Fig. 1).

The dimensions of the paper for the separation into groups are given in Fig. 2.

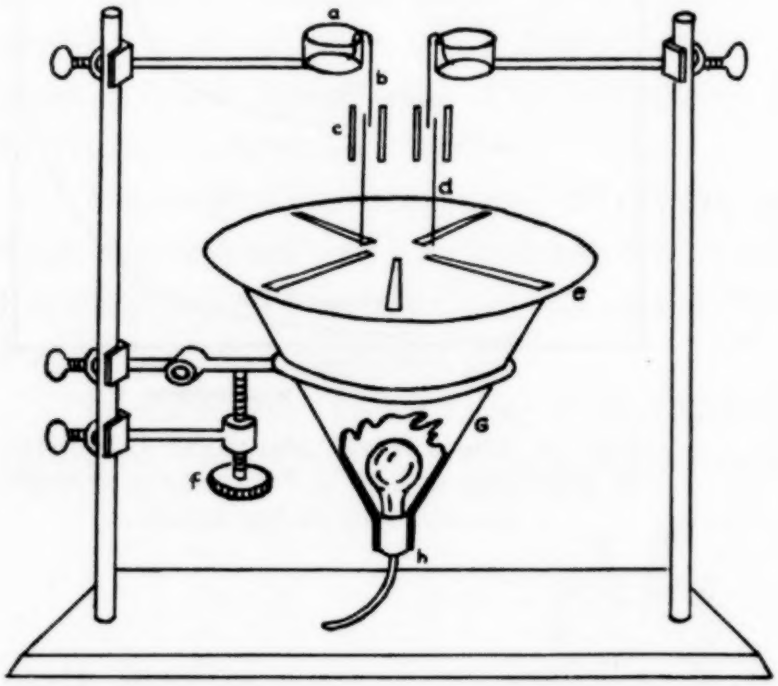

Fig. 1. Simultaneous elution and drying of "group" on K.C.T. paper. (a) Solvent vessel; (b) leader strip; (c) microscope slides; (d) cut out band from first chromatogram; (e) K.C.T. paper; (f) adjustment; (g) light metal cone, base and height 24 cm; (h) 40 W bulb.

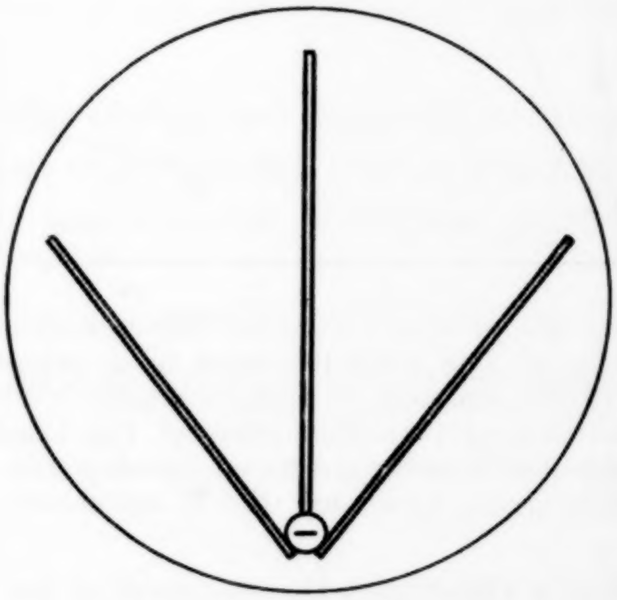

Fig. 2. Circular paper with two segments, one for ninhydrin development the other for cutting out and eluting groups of amino acids. Diameter 33.3 cm, of small circle 2.2 cm; slit in same 2 cm; paper tongue 2 × 5 cm; angles 37° each.

The chromatogram is run with the solvent system *n*-butanol–methyl ethyl ketone–diethylamine–water (20:20:2:10, v/v) which travels about 230 mm within 18 h at 19°. This is very convenient, permitting to start the run in the late afternoon and to take it off next morning.

It was found advisable to increase the size of the box slightly to 37.6 × 37.6 × 7.5 cm and to saturate the atmosphere therein by an additional 40 ml solvent, divided in five regularly spaced, flat dishes. The paper is supported by glass needles of 4 cm length.

Diethylamine should be removed quantitatively in order to avoid complications during the separation of the groups. This is achieved by drying the paper in a ventilated oven at 70° for 30 min and by washing it three times with chloroform.

The distribution of the amino acids and the separation of the various groups with lutidine–water (3:2, v/v) and *tert.*-butanol–formic acid–water (75:0.8:24.2, v/v) are shown in Table I.

TABLE I

GROUP-SEPARATION AND SUB-SEPARATION OF AMINO ACIDS BY HORIZONTAL PAPER CHROMATOGRAPHY

| *Group-separation* | | | *Sub-separation* | |
|---|---|---|---|---|
| *BuOH–Me Et ketone–$H_2O$–diethylamine (20:20:10:2, v/v)* | | | *Lut–$H_2O$ (3:2, v/v)* | *tert.-BuOH–HCOOH–$H_2O$ (75:0.8:24.2, v/v)* |
| *Group* | *amino acids* | $R_F$ | $R_F$ | $R_F$ |
| I | Cystine | 10–16 | | 17–19 |
| | Arginine | | | 23–34 |
| | Asp. acid | | | 40–44 |
| | Glut. acid | | | 50–54 |
| II | Cysteic acid | 18–21 | 44–50 | |
| | Lysine* | | 13–20 | |
| III | Glycine* | 23–25 | 37–41 | |
| | Methiosulfone | | 45–49 | |
| IV | Alanine | 30–37 | | 46–50 |
| | Histidine | | | 18–21 |
| | Serine | | | 38–41 |
| V | Tyrosine* | 42–44 | | |
| VI | Valine | 47–50 | 59–64 | |
| | Methionine | | 48–53 | |
| VII | Threonine | 57–69 | | 45–49 |
| | Phenylalanine | | | 67–70 |
| | Leucine and isoleucine | | | 75–80 |

* Determined immediately after the first run, provided no $H_2O_2$ has been used.

Lysine, glycine and tyrosine appear as single bands and can be determined immediately after the first run. If, however, the sample spot has been treated with a drop of $H_2O_2$, cysteic acid will appear with lysine (group II) and methiosulfone with glycine (group III). Each pair is easily separated by lutidine–water (3:2, v/v) as shown in Table I.

*Fisheries Research Board of Canada, Technological Station,* F. W. van Klaveren*
*Grande-Rivière, Que. (Canada)* G. Vaillancourt

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* Deceased November 3rd, 1959.

# Chromatographic separation of N-2,4-dinitrophenylhydrazides of lower fatty acids

Chromatographic separation of the lower fatty acids has previously been carried out by the author, using a column of Amberlite IRC 50 as adsorbent and a mixture of acetone, methyl ethyl ketone and water as eluent[1]. Since this method did not permit the separation of isobutyric acid from *n*-butyric acid and of α-methylbutyric acid from isovaleric acid, and since the low efficiency of the method seemed to be due to the weaker adsorption of fatty acids on Amberlite IRC 50, the separation of these isomers as their N-2,4-dinitrophenylhydrazides* was investigated.

Amberlite IRC 50 H-form (pulverized and screened as described earlier[1]) was washed on a glass filter with a solvent composed of methyl ethyl ketone, acetone and water (2:1:9 by vol.) and suspended in 2 vols. of the same solvent. The suspension was poured into a chromatographic tube and allowed to settle under gravity. A column of 0.76 cm in diameter and 118 cm in height was used. The N-2,4-dinitrophenylhydrazides were dissolved in solvent of the same composition as that used for the packing of the column and 1 ml of the solution was placed on the column. After the solution had drained into the column, the inner wall of the chromatographic tube was washed with 0.1 to 0.2 ml of the solvent and the N-2,4-dinitrophenylhydrazides were eluted with the same solvent. The effluent was collected in fractions of 3.1 ml in test tubes graduated at 3.5 ml. The flow rate was 4.5 ml/h and the ambient temperature was 28–31°. Each fraction was diluted to 3.5 ml with the solvent used for the chromatographic separation. Then the ultraviolet absorption of each fraction was measured at 340 mμ using a Beckman model DU quartz spectrophotometer (Fig. 1).

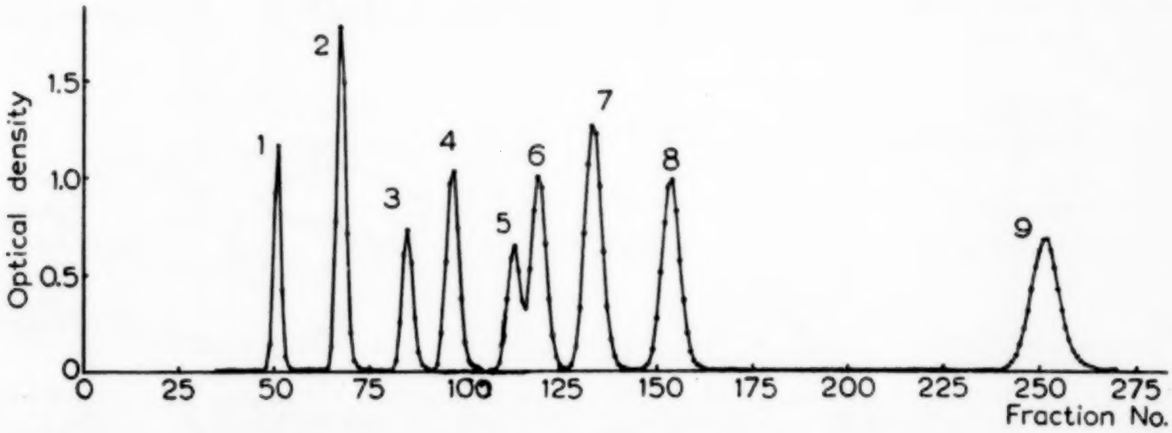

Fig. 1. Elution curve of N-2,4-dinitrophenylhydrazides of lower fatty acids. The compounds in the order of their elution from the column, are: N-2,4-dinitrophenylhydrazide of acetic acid (1), of propionic acid (2), of isobutyric acid (3), of *n*-butyric acid (4), of trimethylacetic acid (5), of α-methylbutyric acid (6), of isovaleric acid (7), of *n*-valeric acid (8) and of caproic acid (9). The N-2,4-dinitrophenylhydrazide of formic acid overlapped that of acetic acid.

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* The N-2,4-dinitrophenylhydrazides were prepared: (1) by boiling 2,4-dinitrophenylhydrazine with excess fatty acid[2] (N-2,4-dinitrophenylhydrazide of formic, propionic and *n*-butyric acids); (2) by boiling 2,4-dinitrophenylhydrazine and fatty acid in 5 *N* sulfuric acid[3] (N-2,4-dinitrophenylhydrazide of trimethylacetic acid); and (3) by dinitrophenylation of fatty acid hydrazide with 2,4-dinitrofluorobenzene[4] (N-2,4-dinitrophenylhydrazide of isobutyric, isovaleric, α-methylbutyric, *n*-valeric and caproic acids). The details will be reported elsewhere.

TABLE I

RECOVERY OF N-2,4-DINITROPHENYLHYDRAZIDES OF LOWER FATTY ACIDS FROM THE CHROMATOGRAPHIC COLUMN

| *N-2,4-Dinitrophenylhydrazide* | *Added (μg)* | *Recovered (μg)* | *Recovery (%)* |
|---|---|---|---|
| Acetic acid | 160 | 168 | 105 |
| Propionic acid | 305 | 320 | 105 |
| Isobutyric acid | 176 | 173 | 98 |
| *n*-Butyric acid | 269 | 272 | 101 |
| Trimethylacetic acid | 204 | 197 | 97 |
| *α*-Methylbutyric acid | 315 | 330 | 105 |
| Isovaleric acid | 461 | 482 | 105 |
| *n*-Valeric acid | 390 | 403 | 103 |
| Caproic acid | 441 | 446 | 101 |

The elution sequence was similar to that of the free fatty acids from a column of Amberlite IRC 50, but the efficiency was increased, that is the two isomers of butyric acid and the four isomers of pentanoic acid were separated as their N-2,4-dinitrophenylhydrazides.

*Department of Genetics, Osaka University Medical School,*
*Osaka (Japan)*

TOKUICHIRO SEKI

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# Chromatographic detection of sugars of growing cartilage

In recent years, the intense metabolic activity of growing cartilage has been extensively investigated[1]; a qualitative and quantitative study of the composition of this tissue seems therefore highly desirable. In the present paper a report is presented of a qualitative examination of the sugar content of growing cartilage.

Investigations were carried out on 40 days old albino rats and on 50 days old rabbits. Cartilage samples were cut out from the proximal end of the "tibia", carefully washed with water and hydrolysed for 10 h in *N*/10 HCl at 100°; 1 ml of acid solution was used per 10 mg of cartilage. The hydrolysate was filtered and evaporated to dryness under vacuum at 50°. The dry residue was repeatedly dissolved in a small amount of water and evaporated to dryness until the pH of the solution was about 5. The residue was then dissolved in a small volume of distilled water and applied to the top of a column (450 × 26 mm) packed with a mixture of equal parts of IMAC A 17 and IMAC C 100 resins (both of Montecatini S.p.A., Milan). The first is a weakly anionic, the second a weakly cationic exchanger. Both were of 80–160 mesh.

The extract was washed out of the column with 150 ml of water at the rate of 0.8 ml/min. The effluent, omitting the dead volume, was collected in a container containing 1–2 ml toluene and evaporated to dryness under vacuum at 50°. The residue was dissolved in 0.5 ml of water saturated with benzoic acid. In order to obtain a complete recovery of the extract, the walls of the vessel were repeatedly washed with hot methanol. The aqueous solution and the methanol washings were united and evaporated to about 0.5 ml under a current of dry air.

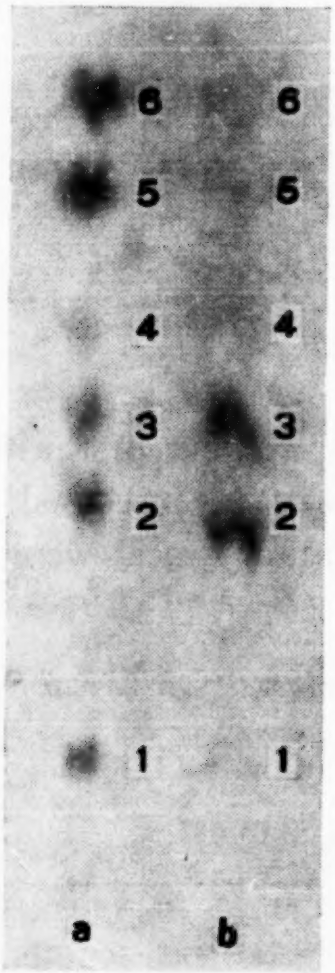

Fig. 1. Paper chromatography of sugars of growing cartilage. Solvent: *n*-butanol–pyridine–benzene–water (5:3:1:3)[2]. Details of the procedure are reported in the text. a = reference mixture of pure sugars; b = cartilage hydrolysate. (1) lactose, (2) galactose, (3) glucose, (4) fructose, (5) xylose, (6) ribose.

0.2 ml of the concentrated fluid (corresponding to about 100 mg of fresh tissue) was chromatographed on Whatman No. 1 paper using *n*-butanol–pyridine–benzene–water (5:3:1:3) as solvent according to WHITE AND HESS[2], and the sugar spots were detected by spraying the chromatogram with a mixture (1:1, v/v) of a 2 % solution of double distilled aniline in ethanol and 0.2 *M* citric acid[2]. After drying, the paper was kept for a few minutes at 100°.

A typical chromatogram is reproduced in Fig. 1.

The identification of the sugars was carried out by comparison with a known mixture of chemically pure sugars (see Fig. 1) and by separate addition of pure samples of each of the sugars to the extract.

Identical results were obtained with rats and rabbits. As evident from the described procedure, these results concern total sugar of growing cartilage, *i.e.* free sugars and sugars arising from acid hydrolysis of the polysaccharides.

This work was supported by a grant from Consiglio Nazionale delle Ricerche.

*Institute of Biological Chemistry, University of Padua (Italy)*
F. PETRELLI
N. SILIPRANDI
L. SARTORELLI

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## BOOK REVIEWS

*Comprehensive Analytical Chemistry*, Volume IA, *Classical Analysis*, edited by CECIL L. WILSON AND DAVID W. WILSON, Elsevier Publishing Company, Amsterdam, 1959, xx + 577 pages, price £ 5.5.0.

The success of RODD'S *"Chemistry of Carbon Compounds"*, which is one of the best texts on organic chemistry, has induced the publishers to promote another very important comprehensive work, this time in the field of analytical chemistry, *viz.* *"Comprehensive Analytical Chemistry"*, edited by CECIL WILSON AND DAVID WILSON.

The first volume of this work deals with classical analysis and part IA of this volume, which appeared recently, comprises six chapters, the first a general introduction, the others devoted to analytical processes, gas analysis, inorganic qualitative analysis, organic qualitative analysis, inorganic gravimetric analysis.

It must be recognized that the task undertaken by the Editors is very difficult owing to the vastness of this work.

A first observation must be made: *"Comprehensive Analytical Chemistry"* should be regarded more as a source of information and literature rather than as a book to be used in the laboratory when performing an analysis in practice.

Only with this in mind can it be understood why, for instance, only two pages are dedicated to countercurrent distribution.

The authors of the different sections have certainly been well chosen, but notwithstanding this the sections do not all appear to be of the same high standard, *e.g.* the treatment of qualitative organic analysis is rather inadequate, no mention being made of systematic methods; in the case of inorganic qualitative analysis it is a pity that the systematic classical separation (according to TREADWELL) has not been mentioned at all, although this volume is specifically devoted to classical analysis. Apart from these shortcomings, understandable owing to the huge task of the Editors, this book is of a high standard, and for this reason it will be welcomed by all people interested in the progress of chemistry. It can be said that it will constitute a fundamental text in the field of chemical literature.

g.b.m.b. (Rome)

*Gaschromatographie*, by ERNST BAYER, Vol. X of *Anleitungen für die Laboratoriumspraxis*, Springer Verlag, Berlin, 1959, iv + 163 pages, price DM 39.60.

The aim of this book published in Springer Verlag's "*Anleitungen für die Laboratoriumspraxis*" is to provide a practical introduction to the field of gas chromatography. It is divided into four parts:

Part I describes very briefly the theoretical principles underlying gas chromatography and gives only basic concepts. Part II is dedicated to the nature and composition of the columns and Part III to instrumentation: these subjects are treated concisely but adequately for the purpose of the book. Part IV is devoted to the practical applications of gas chromatography. Here the author gives a detailed presentation of the results that have been obtained for various classes of substances and in technical analysis. This part is well organised, carefully planned and gives a broad, but not complete, coverage of most of the experimental data up to 1958.

The field of gas chromatography is developing so rapidly that any book written on this topic soon becomes out of date. For instance, the use of capillary columns, their amazing achievements and their revolutionary influence on instrumentation are only mentioned.

However, this volume fills a long felt need for a simple and easy-to-read book, and it is an exceedingly useful tool for the unskilled beginner and for the qualified gas chromatography technician.

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# 16α-HYDROXY STEROIDS

## III. RECOGNITION OF THE 16α,17α-DIOL FEATURE OF TRIAMCINCOLONE BY CYCLIC KETAL FORMATION ON PAPERGRAMS

LELAND L. SMITH AND THEODORE FOELL
*Chemical Process Improvement Department, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y. (U.S.A.)*

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### INTRODUCTION

In studies of microbiological 16α-hydroxylation of steroids bearing the dihydroxyacetone side chain it was necessary to establish the presence of the anticipated 16α,17α-diol feature by definitive means with very small amounts of sample. Although mobility behavior in several paper chromatographic systems serves adequately for recognition of known steroids, other evidence is required when dealing with steroids not previously characterized. Of the auxiliary techniques commonly employed to support papergram identifications—derivative formation (acetylation, oxidation), elution and spectral studies, bioassay, etc.—none was considered sufficiently specific for our purposes. Of the specific reactions of 16α,17α,21-trihydroxy-20-ketones the formation of 16α,17α-cyclic ketals[1,2] was selected as being both specific for the 16α,17α-diol feature and as being adaptable to reaction on the very paper which later serves for papergram analysis.

This report deals with the development and application of the method of forming 16α,17α-cyclic ketals and acetals *in situ* on papergrams for the early recognition of the 16α,17α-diol feature of triamcinolone (9α-fluoro-11β,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20-dione) and related 16α-hydroxylated steroids.

### EXPERIMENTAL

Paper chromatographic separations of the steroids and steroid ketal (acetal) derivatives formed in the reaction studies were made using the solvent systems already described[3]. Systems V and VI were used for analysis of the ketals and acetals formed; System II was used for the analysis of unaltered steroid alcohols. The usual precautions and techniques for operation of Bush-type systems were carefully observed[4]. The finished chromatograms were examined for ultraviolet absorbing zones, for unsaturated 3-ketosteroid zones (*via* isonicotinic acid hydrazide) and for reducing α-ketol zones (*via* alkaline tetrazolium blue) in the manner already described[3,5].

The steroids used were of high purity as evidenced by papergram behavior, ultraviolet absorption spectra, and infrared absorption spectra. 16α,17α-Cyclic ketal and acetal derivatives used as reference materials were prepared by the method of FRIED[1]. All steroids were applied at 20 $\mu$g levels to sheets of unwashed Whatman No. 1 filter paper as 1 mg/ml solutions in absolute methanol.

Cyclic ketal formation on paper was promoted by spotting a suitable volume (20–40 $\mu$l) of diluted perchloric acid (0.3 ml of reagent 70% perchloric acid diluted to 50 ml with either acetone or with methanol) directly over the dried steroid spot previously applied. The area covered by the diluted acid was purposely made larger than that of the steroid zone so as to assure complete reaction of all of the steroid. The acetone-diluted reagent was used for acetonide formation, the methanol-diluted reagent for other ketals and for acetals. The treated paper was then hung in a closed chamber saturated with vapors of the carbonyl compound to be used. Saturation was assured by a layer of the liquid ketone in the bottom of the chamber. The chamber was kept at 29°, the same temperature at which the chromatogram was developed. After a suitable time interval of exposure to the carbonyl compound, the paper was removed, dried momentarily in air and developed chromatographically.

A standard reaction condition was established using 20 $\mu$g of steroid, 30 $\mu$l of diluted perchloric acid and exposure to acetone for 16 h. These conditions are sufficient for complete conversion of the 16α-hydroxy steroids studied to their respective 16α,17α-acetonides.

The treated paper is equilibrated and developed in the system of choice and the products formed of the reaction are visualized and marked. Suitable reference compounds of the anticipated cyclic ketal are run along with the experimental sample. By inspection the identification of a reaction product ketal with a reference known ketal can be made, and thus the presence of the 16α,17α-diol feature surmised. In no case was the mobility of the derivative formed on paper different from that of an authentic reference derivative.

Control experiments were run with each steroid used. Each sample was:
(a) treated with acid, held for 16 h in air, but not exposed to acetone,
(b) not treated with acid but exposed to acetone for 16 h, and
(c) treated with acid and exposed to acetone for 16 h.

These control experiments were performed on both 16α-hydroxy and non-16α-hydroxy steroids. Each experiment was run in at least two different chromatographic systems.

Confirmation of acetonide formation with triamcinolone was made by streaking 200 $\mu$g of triamcinolone across a 7-inch sheet of paper. Seven such sheets were prepared. Over each steroid zone was streaked 150 $\mu$l of a solution of 0.30 ml of 70% perchloric acid diluted with 25 ml of acetone. The sheets were exposed to acetone vapors for 16 h, then equilibrated and run in System VI[3]. The steroid zone was located by ultraviolet light absorption, eluted with hot acetone, the eluted material concentrated and chromatographed over Florisil (adsorbed from benzene solution), reconcentrated and rechromatographed on two 7-inch sheets of filter paper using System V[3]. The steroid zone was located by cutting a small strip from the center of the sheets and visualizing with isonicotinic acid hydrazide and tetrazolium blue. The steroid zone was eluted with hot acetone, concentrated *in vacuo*, and the crystalline precipitate was recrystallized from methanol and then from acetone. Comparison of infrared absorption spectra of *ca.* 100 μg of the sample in a potassium bromide disk over the range 2–15 μ and of sulfuric acid absorption spectra[6] over the range 220–600 mμ established the identity of the sample with that of authentic triamcinolone 16α,17α-acetonide.

## RESULTS

The reaction on papergrams of a variety of 16α,17α,21-trihydroxy-20-ketosteroids with a variety of carbonyl compounds to form the 16α,17α-cyclic ketal or acetal is evidenced in Tables I and II. Each 16α-hydroxy steroid examined afforded its respective 16α,17α-cyclic derivative under reproducible conditions and with no detectable other products. Although ketal (acetal) formation was not complete in all cases, only the anticipated product was found together with unaltered parent steroid. Using the standard conditions of 16 h of exposure to acetone, complete reaction with 6 representative 16α-hydroxy steroids was accomplished, with no artifacts of degradation, rearrangement, dehydration, etc. encountered. A high order of confidence was established for the reaction of 16α-hydroxy steroids with acetone under the standard conditions.

Under control condition (a) both 16α-hydroxyhydrocortisone and 9α-fluoro-16α-hydroxyhydrocortisone formed traces of artifacts of an undetermined nature. These artifacts were not present in the pure untreated steroid sample and they were not found under the standard reaction conditions. Their presence under control condition (a) was not of further concern. The papergram mobilities of steroids treated under control condition (a) were not affected by the treatment, the same $R_F$ values being obtained for reference steroids and for derivatives formed on paper.

Control condition (b) did not have any effect on migration, color tests, etc. of any steroid studied.

Long exposure (64 h) of other 16α-hydroxy steroids to control condition (a) gave the same indications of traces of artifact formation, as did exposure of non-16α-hydroxylated steroids to these conditions. It is important to limit the exposure of treated steroids on paper to 16 h and even then it may be necessary to discount traces of artifacts in certain cases. In no instance did artifact formation appear except in trace amounts, and it is only with the very sensitive isonicotinic acid hydrazide and tetrazolium blue reagents that they may be detected at all.

Whereas the reaction between 16α,17α-diols and acetone is complete in a relatively short time, other ketones and acetaldehyde do not react completely in short times, and in some cases not at all (Table II). Although 9α-fluoro-16α-hydroxyhydrocortisone formed a ketal (acetal) in each case, its 1-dehydro analog, triamcinolone, failed to form a derivative with methyl isobutyl ketone even after 64 h of exposure. Throughout the study the impression was obtained that 16α,17α-diol $\Delta^4$-ketosteroids reacted more readily than their respective 1-dehydro analogs. No experiments were run at elevated temperature to promote reaction.

TABLE I

COMPARISON OF RESULTS FROM REACTION WITH ACETONE (STANDARD CONDITIONS) OF 16α-HYDROXYLATED AND NON-16α-HYDROXYLATED STEROIDS

| *Steroid parent* | *Parent non-16α-hydroxylated steroid* | | | *16α-Hydroxylated steroid analog* | | |
|---|---|---|---|---|---|---|
| | *System* | *$R_F$* | *Identity*[*,**] | *System* | *$R_F$* | *Identity*[*,***] |
| Hydrocortisone (F) | V | 0.07 | F | V | 0.58 | F 16 IP |
| | VI | 0.01 | F | VI | 0.40 | F 16 IP |
| Prednisolone ($\Delta^1$-F) | II | 0.77 | $\Delta^1$-F | V | 0.44 | $\Delta^1$-F 16 IP |
| | VI | 0.0 | $\Delta^1$-F | VI | 0.29 | $\Delta^1$-F 16 IP |
| 9α-Fluorohydrocortisone (9αFF) | II | 0.80 | 9αFF | II | 0.95 | 9αFF 16 IP |
| | V | 0.02 | 9αFF | V | 0.49 | 9αFF 16 IP |
| 9α-Fluoroprednisolone (9αF$\Delta^1$-F) | II | 0.75 | 9αF$\Delta^1$-F | II | 0.88 | 9αF$\Delta^1$-F 16 IP |
| | VI | 0.0 | 9αF$\Delta^1$-F | V | 0.34 | 9αF$\Delta^1$-F 16 IP |
| | | | | VI | 0.15 | 9αF$\Delta^1$-F 16 IP |
| Reichstein's Substance S (S) | II | 0.93 | S | V | 0.94 | S 16 IP |
| | V | 0.44 | S | VI | 0.90 | S 16 IP |
| 9(11)-Dehydro Substance S ($\Delta^{9(11)}$-S) | V | 0.40 | $\Delta^{9(11)}$-S | V | 0.92 | $\Delta^{9(11)}$-S 16 IP |
| | VI | 0.18 | $\Delta^{9(11)}$-S | VI | 0.91 | $\Delta^{9(11)}$-S 16 IP |

[*] Abbreviations used throughout the tables are: F, hydrocortisone; F 16, 16α-hydroxyhydrocortisone; 9αFF, 9α-fluorohydrocortisone; 9αFF 16, 9α-fluoro-16α-hydroxyhydrocortisone; $\Delta^1$-F, prednisolone; S, Reichstein's Substance S; S 16, 16α-hydroxy Substance S; IP, 16α,17α-cyclic acetonide (isopropylidenedioxy); etc.

[**] Only the parent steroid was detected, with no trace of any other component.

[***] Only the anticipated 16α,17α-acetonide was detected, with no trace of any other component.

TABLE II

REACTION OF 16α-HYDROXYLATED STEROIDS WITH CARBONYL COMPOUNDS OTHER THAN ACETONE

| *Carbonyl compound* | *Steroid* | *Reaction conditions* | | *Components found* | | | *Comments* |
|---|---|---|---|---|---|---|---|
| | | *Acid volume, ul* | *Exposure time, h* | *System* | $R_F$ | *Identity** | |
| Acetaldehyde | 9αFF 16 | 30 | 16 | II | 0.92 | 9αFF 16 ED | Incomplete reaction under standard conditions |
| | | | | | 0.57 | 9αFF 16 | |
| | | | | VI | 0.22 | 9αFF 16 ED | |
| | | | | | 0.0 | 9αFF 16 | |
| | 9αF$\Delta^1$-F 16 | 30 | 16 | II | 0.93 | 9αF$\Delta^1$-F 16 ED | Incomplete reaction under standard conditions |
| | | | | | 0.45 | 9αF$\Delta^1$-F 16 | |
| | | | | VI | 0.12 | 9αF$\Delta^1$-F 16 ED | |
| | | | | | 0.0 | 9αF$\Delta^1$-F 16 | |
| Methyl ethyl ketone | 9αFF 16 | 20 | 16 | V | 0.58 | 9αFF 16 MEK | Complete reaction; no other products |
| | | | | VI | 0.42 | 9αFF 16 MEK | |
| | 9αF$\Delta^1$-F 16 | 30 | 64 | V | 0.45 | 9αF$\Delta^1$-F 16 MEK | Incomplete reaction at 16 h |
| | | | | VI | 0.28 | 9αF$\Delta^1$-F 16 MEK | Complete at 64 h |
| Methyl isobutyl ketone | 9αFF 16 | 40 | 40 | VI | 0.64 | 9αFF 16 MIBK | Incomplete reaction under several conditions. Very faint other components formed |
| | | | | | 0.0 | 9αFF 16 | |
| | 9αF$\Delta^1$-F 16 | 40 | 64 | II | 0.47 | 9αF$\Delta^1$-F 16 | No reaction observed between steroid and MIBK; some isomerization observed |
| | | | | | 0.25 | Triamcinolone isomer | |
| | | | | VI | 0.0 | 9αF$\Delta^1$-F 16 | |

* Abbreviations: ED, 16α,17α-ethylidenedioxy; MEK, 1-methylpropylidenedioxy; MIBK, 1,2,2-trimethylpropylidenedioxy. Other abbreviations listed in footnote* of Table I.

The reactions of some other steroid 1,2-diols and/or 1,3-diols are listed in Table III.

Treatment of the filter paper with the dilute perchloric acid reagent caused no variation in mobility of steroid components in the three Bush-type systems used. The paper is altered in its properties only where the acid is spotted, and a large whitish spot is seen at the origin after color development on the finished chromatogram. The perchloric acid spot does not interfere with detection by ultraviolet absorption or by isonicotinic acid hydrazide fluorescence. Detection of immobile reducing steroids with tetrazolium blue is not possible. In no case did the acid treatment interfere with visualization procedure once the steroid had migrated off the origin position.

TABLE III

REACTION WITH ACETONE (STANDARD CONDITIONS) OF OTHER STEROID DIOLS

| *Steroid diol* | *Components found* | | | *Comments* |
|---|---|---|---|---|
| | *System* | $R_F$ | *Assigned identity** | |
| Triamcinolone isomer | II | 0.88 | IP derivative | Partial reaction; reaction also incomplete after 64 h |
| | | 0.25 | Isomer | |
| | VI | 0.02 | Isomer and IP derivative | |
| 1,2-Dihydrotriamcinolone isomer | II | 0.89 | Isomer IP | Partial reaction |
| | | 0.30 | Isomer | |
| 20,20-Dihydro-9α-fluoro-hydrocortisone | II | 1.0 | IP derivative | Partial reaction. No reference samples used |
| | | 0.42 | 20-$H_2$9αFF | |
| | VI | 0.25 | IP derivative | |
| | | 0.0 | 20-$H_2$9αFF | |
| 16α,17α-Dihydroxy-progesterone | V | 1.0 | IP derivative | Complete reaction indicated, with no unaltered diol (at $R_F$ 0.61 and 0.46 in Systems V and VI) |
| | VI | 1.0 | IP derivative | |
| 14α-Hydroxy Substance S | VI | 0.30 | 14-Dehydro S | Dehydration rather than acetonide formation |
| | | 0.12 | 14α-Hydroxy S | |

* Abbreviations: IP, cyclic acetonide; 20-$H_2$, 20,20-dihydro-.

Where the acetone-diluted perchloric acid reagent was compared with the methanol-diluted reagent, no effect was noticed with 16α,17α-diols for acetonide formation. However, use of the acetone-diluted reagent for some other ketal reactions gave some traces of what would appear to be the cyclic acetonide (from relative migration measurements) together with the major ketal product formed from reaction with ketone vapors. These trace artifacts were not examined further.

## DISCUSSION

Although many chemical reactions of steroids have been run on filter paper, their use has been confined to purposes of visualization of resolved steroids after chromatographic irrigation. That this restriction need not be the case is established by the results of the present study, as it is readily apparent that select chemical reactions of steroids may be accomplished on paper prior to chromatography.

The formation of 16α,17α-cyclic ketals on filter paper and subsequent analysis is an especially favorable situation, both from a viewpoint of use of volatile ketones to afford simple reaction conditions and from the viewpoint of the particularly reactive 16α,17α-diol feature of the steroids studied.

The further application of the volatile reactant-reaction on filter paper principle should be considered in such cases as acetylations, hydrolyses, brominations, and catalytic reductions. Catalytic reduction of unsaturated fatty acids on filter paper has already been reported; the saturated fatty acids so produced were then resolved chromatographically on the same paper[7].

The procedure is reliable for the early recognition of the *cis*-diol feature, particularly of the especially reactive 16α,17α-diol feature, in routine microbiological screening programs. It is currently accepted that *cis*-1,2-diols and 1,3-diaxial diols may form cyclic acetonides[8,9]. Thus other steroid diols which form cyclic acetonides could be mistakenly recognized as 16α,17α-diols using the procedure in unknown situations.

Under the standard conditions with acetone certain other steroids diols (see Table III) do indeed form more mobile derivatives on papergrams, such derivatives being tentatively identified as the respective cyclic acetonide. In the few cases studied complete reaction was not attained, and considerable unaltered diol was found, in distinction to the complete reaction encountered with established 16α,17α-diols.

20,20-Dihydro-9α-fluorohydrocortisone[10] would be expected to form a 20,21-cyclic acetonide[11, 12], and 16α,17α-dihydroxyprogesterone should also form a 16α,17α-acetonide. In both cases more mobile components were noted, but no effort was made to establish the precise nature of the product formed. Mobility and color test behavior of the products is consistent with a cyclic acetonide formulation. It should be pointed out that 16α,17α-dihydroxyprogesterone was completely converted to more mobile material whereas 20,20-dihydro-9α-fluorohydrocortisone was but partially converted.

In the instance of triamcinolone isomer and 1,2-dihydrotriamcinolone isomer[13], partial reaction to form the acetonide occured. Comparison of the product formed from triamcinolone isomer with authentic triamcinolone isomer cyclic acetonide[14] indicated identity. These rearrangement products of the parent steroids also form derivatives with acetaldehyde and methyl ethyl ketone on paper, and again the $\Delta^{1,4}$-3-ketone, triamcinolone isomer, is more sluggish in derivative formation than its $\Delta^{4}$-3-ketone analog, 1,2-dihydrotriamcinolone isomer. These isomeric steroids are suspected of being 16α-hydroxylated D-homoannulated structures[14] and as such should form cyclic acetonides[15, 16].

The one case of a known 1,3-diaxial diol in Table III involves 14α-hydroxy Reichstein's Substance S (14α,17α,21-trihydroxy-4-pregnene-3,20-dione). The product obtained, together with apparently unaltered steroid, was slightly less mobile than Substance S, and therefore cannot be the postulated 14α,17α-cyclic acetonide, but rather is more likely the 14-dehydro derivative, 17α,21-dihydroxy-4,14-pregnadiene-3,20-dione. Substance S and the 16α,17α-cyclic acetonide of 9α-fluoro-16α-hydroxyhydrocortisone have approximately the same mobility in the system used, and the anticipated mobility for the postulated 14α,17α-cyclic acetonide should be substantially greater than that of Substance S, rather than slightly inferior. The known acid dehydration of 14α-hydroxy Substance S to the 14-dehydro derivative would also support this concept[17, 18].

Despite the possible confusion of potential acid-induced rearrangements with or without subsequent derivative formation, of partial reaction effects, of dehydration effects, reaction at other diol sites, etc. all of which tend to compromise the full reliability of the procedure in unknown situations, strict adherence to standard conditions, together with the proper use of related, known reference compounds, will permit differentiation of 16α,17α-diols from other types. In certain cases some modification of the standard conditions might permit a similar specific application to other microbiological screening programs where other diols capable of cyclic acetonide formation are sought, as in recent microbiological 12β-hydroxylation of 11β-hydroxy steroids[19].

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## SUMMARY

A procedure for the preparation of 16α,17α-cyclic acetonide derivatives of steroid 16α,17α-diols directly on paper chromatograms is described. After reaction on paper under standard conditions the acetonides formed are resolved by chromatographic development of the paper and the separated components are visualized. The procedure is suggested for early recognition of the 16α,17α-diol feature of certain steroid molecules.

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# PAPER CHROMATOGRAPHY AND STRUCTURAL RELATIONSHIPS OF ORGANIC ACIDS

J. R. HOWE
*Low Temperature Research Station, Cambridge (Great Britain)*

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## INTRODUCTION

An equation relating the partition coefficient of a solute between two liquid phases to its $R_F$ value on paper was postulated by CONSDEN, GORDON AND MARTIN[1] in 1944:

$$\alpha = \frac{A_L}{A_S}\left(\frac{1}{R_F} - 1\right) \qquad (1)$$

where $\alpha$ = partition coefficient of solute between the stationary and moving phases, and $A_L$ and $A_S$ are the cross sectional areas of the moving and stationary phases respectively.

Following on from this in 1949, MARTIN[2] derived a relationship in which the free energy required to transport a molecule from one "ideal" liquid phase to another was proportional to the logarithm of the partition coefficient of the solute between the two phases. He then made two simplifying assumptions:

(1) that the total free energy of a molecule is composed of the sum of the free energies of the constituents composing the molecule; thereby implying that all isomers containing the same constituents would have the same partition coefficients;

(2) that the free energy required to transfer a given constituent (*e.g.* $-CH_2-$) of the molecule, from one solvent to another, is independent of the remainder of the molecule.

From this he was able to predict that the difference between the logarithms of the parition coefficients for adjacent members of a homologous series would be constant provided that the degree of ionisation of the members was the same. Therefore from equation (1) above, if $A_L/A_S$ is constant over the whole paper, log $(1/R_F - 1)$ should decrease by equal steps as the number of substituents is increased in a homologous series.

In order to test this proposition BATE-SMITH AND WESTALL[3] introduced the term "$R_M$ value" such that

$$R_M = \log\left(\frac{1}{R_F} - 1\right) \qquad (2)$$

and found practically constant $\Delta R_M$ values for the introduction of hydroxyl and glucose groups into a number of aromatic compounds. The relationship was further investigated for organic acids by ISHERWOOD AND HANES[4] in a range of propanol–

ammonia solvents. They found $\Delta R_M$ constant for unit increase of $n$ in the homologous dicarboxylic acid series $HOOC\cdot(CH_2)_n\cdot COOH$, but with the monocarboxylic acids $H\cdot(CH_2)_n\cdot COOH$ the $\Delta R_M$ values for unit addition of $-CH_2-$ decreased.

In the present paper, this type of investigation has been extended to a large number of organic acids in which several homologous series are included. The $R_M$ values of the acids are compared, both in an alkaline solvent where the acids were ionised and also in an acidic solvent where the ionisation of the carboxyl groups of the acids was suppressed. By relating the $R_M$ values from the two solvents it is possible to predict the number, type and configuration, of many of the substituent groups present in the acid molecule.

The literature on paper chromatography contains numerous examples of factors which affect the $R_F$ value. These factors include temperature, purity of solvents, equilibration, volume of solvent relative to volume of chromatogram tank, ascending or descending solvent, distance travelled by solvent, type of paper, distance of point of application of spot from solvent level, pH of solution applied to the base-line, etc. Although the individual $R_F$ values may vary with each of these factors, the overall chromatographic pattern of spots is likely to be similar no matter what conditions are chosen provided that a standardised procedure is adopted. Therefore the experimental conditions actually used in this paper are described in some detail in the next section and were always followed, very closely, for each set of determinations of $R_F$ values.

## MATERIALS AND METHOD

Chromatogram jar, dimensions: 29 × 19 × 45 cm.
Whatman No. 1 filter paper, dimensions: 23 × 45 cm.
Alkaline solvent: *n*-propanol–2 *N* aqueous ammonia (70:30).
Acid solvent: *n*-propanol–water saturated with sulphur dioxide (70:30).
Spray for acids: 15 ml Universal Indicator (B.D.H.) + 2–3 ml 0.1 *N* sodium hydroxide[5].
Spray for amino acids: 0.4% ninhydrin + 0.2% cobalt chloride + 5% water in isopropyl alcohol[6].
Spray for neutral compounds: 0.2 *N* silver nitrate + 880 ammonia (6:1)[7].

Whatman No. 1 filter papers were washed prior to use with 2 *N* aqueous acetic acid, followed by distilled water and then 10 *N* aqueous ammonia solution as described by ISHERWOOD AND HANES[4]. All acids which were to be revealed later by a pH indicator were prepared as 0.1 or 0.2 *N* solutions and those to be revealed by ninhydrin were at 0.05 *M* strength. Within each homologous series all the individual members were of the same normality and all acids were dissolved in 2 *N* ammonium hydroxide solution. 4 $\mu$l spots of the solutions of the ammonium salts were applied on a starting line drawn 7.5 cm from one end of the paper and the papers were then hung in the jars for descending chromatography.

The reagents used in the preparation of solvents were the purest commercial grades available and no further purification was attempted. Solvents were always prepared accurately as small variations, particularly in water content, had a significant effect on $R_F$ value. This is important because the derived relationships depend on the fact that the final solvents were always of the same composition. The equilibration liquid, which consisted of 200 ml of either the acidic or the basic solvent, was splashed down the sides of the jar and the papers were gently flapped inside the jar for 2 h by the method described by HANES AND ISHERWOOD[8]. Solvent was then added to the trough and allowed to descend down the paper for about 10 h in which time the solvent front had advanced approximately 27 cm beyond the starting line. Chromatography was carried out at a constant temperature of $20° \pm 0.5°$.

When the papers were sprayed with Universal Indicator, the acid spots appeared immediately and their exact position was outlined because the contrast between the spot and background was usually greatest at this stage. These chromatograms can be retained as permanent records if stored away from acidic or basic vapours.

## RESULTS AND DISCUSSION

Chromatography was carried out under the above conditions and the results are presented in Table I as $R_F$ and $R_M$ values for each solvent. In order to emphasise the structural relationship of these acids they are arranged in groups, in the Table, primarily according to whether they are mono-, di-, tri- or tetra-carboxylic acids. Each carboxyl grouping of acids is subdivided into the substituted groups, *e.g.* hydroxyl, amino etc. and finally the acids themselves are placed in homologous series, where possible, followed by any other acids of similar substitution in order of increasing carbon number. To assist in the comparison of related compounds a separate column is devoted to the number of carbon atoms in each acid. All the compounds in Table I have been numbered consecutively from 1 to 111 and these numbers are used in the figures and text.

### *Solvents*

The solvents used were chosen as a result of the following considerations:

(a) In order to restrict the number of variables, systems of 1 organic component only were considered, *i.e.* 1 organic component + dilute aqueous acid (or alkali). Furthermore it was considered desirable to have the two solvents as similar in composition as possible, differing from one another only in the small amount of acid or base which had to be added.

(b) A large difference was required between the $R_F$ values of each acid in the two solvents so that the effect of the –COOH group was accentuated. In a series of investigations with solvents containing alcohols it was found that this difference increases with the chain length of the alcohol but is limited by the low solubility of water in the higher alcohols. In solvents with low water contents the salts tend to streak and with the longer chain alcohols the di-, tri- and tetra-carboxylic acids in the alkaline solvent do not move from the base line and therefore cannot be distinguished from one another. This meant that propanol was the highest chain length

TABLE I

$R_F$ AND $R_M$ VALUES OF ACIDS AND SOME NEUTRAL COMPOUNDS

| *Code No.* | *Carbon No.* | *Compound* | *n-Propanol–2 N ammonia (70:30)* | | *n-Propanol–water-sat. $SO_2$ (70:30)* | | *$(R_M)$ alkaline —$(R_M)$ acid* |
|---|---|---|---|---|---|---|---|
| | | | $R_F$ | $R_M$ | $R_F$ | $R_M$ | |
| | | MONOCARBOXYLIC ACIDS | | | | | |
| | | *Unsubstituted fatty acids* | | | | | |
| 1 | 1 | Formic | 0.47 | + 0.05 | Acids too volatile to record | | |
| 2 | 2 | Acetic | 0.48 | + 0.03 | | | |
| 3 | 3 | Propionic | 0.56 | — 0.10 | | | |
| 4 | 4 | *n*-Butyric | 0.62 | — 0.21 | | | |
| 5 | 5 | *n*-Valeric | 0.67 | — 0.31 | | | |
| 6 | 6 | Caproic | 0.73 | — 0.43 | 0.90 | — 0.95 | 0.52 |
| 7 | 7 | Heptanoic | 0.76 | — 0.50 | 0.91 | — 1.00 | 0.50 |
| 8 | 8 | Caprylic | 0.79 | — 0.57 | 0.92 | — 1.06 | 0.49 |
| 9 | 9 | Nonanoic | 0.80 | — 0.60 | 0.92 | — 1.06 | 0.46 |
| 10 | 10 | Decanoic | 0.81 | — 0.63 | 0.93 | — 1.12 | 0.49 |
| 11 | 12 | Lauric | 0.81 | — 0.63 | 0.93 | — 1.12 | 0.49 |
| 12 | 14 | Myristic | 0.81 | — 0.63 | 0.93 | — 1.12 | 0.49 |
| 13 | 4 | Isobutyric | 0.61 | — 0.19 | Acids too volatile to record | | |
| 14 | 5 | Isovaleric | 0.66 | — 0.29 | | | |
| | | *Mono-hydroxy acids* | | | | | |
| 15 | 2 | Glycollic | 0.38 | + 0.21 | 0.69 | — 0.35 | 0.56 |
| 16 | 3 | Lactic | 0.47 | + 0.05 | 0.77 | — 0.52 | 0.57 |
| 17 | 4 | 2-Hydroxy-*n*-butyric | 0.54 | — 0.07 | 0.82 | — 0.66 | 0.59 |
| 18 | 5 | 2-Hydroxy-*n*-valeric | 0.62 | — 0.21 | 0.85 | — 0.75 | 0.54 |
| 19 | 6 | 2-Hydroxy-caproic | 0.68 | — 0.33 | 0.88 | — 0.86 | 0.53 |
| 20 | 8 | 2-Hydroxy-caprylic | 0.76 | — 0.50 | 0.90 | — 0.95 | 0.45 |
| 21 | 15 | 2-Hydroxy-pentadecanoic | 0.78 | — 0.55 | 0.91 | — 1.01 | 0.46 |
| 22 | 4 | 3-Hydroxy-*n*-butyric | 0.50 | 0.00 | 0.79 | — 0.57 | 0.57 |

(*continued on p. 393*)

TABLE I (*continued*)

| Code No. | Carbon No. | Compound | *n-Propanol-2 N ammonia (70:30)* | | *n-Propanol-water-sat. SO₂ (70:30)* | | $(R_M)$ *alkaline* —$(R_M)$ *acid* |
|---|---|---|---|---|---|---|---|
| | | | $R_F$ | $R_M$ | $R_F$ | $R_M$ | |
| | *Di-hydroxy acids* | | | | | | |
| 23 | 3 | Glyceric | 0.37 | + 0.23 | 0.62 | — 0.21 | 0.44 |
| | *Mono-amino acids* | | | | | | |
| 24 | 2 | Glycine | 0.31 | + 0.35 | 0.42 | + 0.14 | 0.21 |
| 25 | 3 | α-Alanine | 0.39 | + 0.19 | 0.56 | — 0.10 | 0.29 |
| 26 | 4 | α-Amino-*n*-butyric | 0.47 | + 0.05 | 0.63 | — 0.23 | 0.28 |
| 27 | 5 | Norvaline | 0.58 | — 0.14 | 0.71 | — 0.39 | 0.25 |
| 28 | 6 | Norleucine | 0.68 | — 0.33 | 0.77 | — 0.53 | 0.20 |
| 29 | 8 | α-Amino-caprylic | 0.75 | — 0.48 | 0.82 tail | — 0.66 | 0.18 |
| 30 | 3 | β-Alanine | 0.34 | + 0.29 | 0.50 | 0.00 | 0.29 |
| 31 | 4 | γ-Amino-butyric | 0.36 | + 0.25 | 0.54 | — 0.07 | 0.32 |
| 32 | 6 | ε-Amino-caproic | 0.46 | + 0.07 | 0.65 | — 0.27 | 0.34 |
| 33 | 8 | ω-Amino-caprylic | 0.63 | — 0.23 | 0.75 | — 0.48 | 0.25 |
| 34 | 4 | α-Amino-isobutyric | 0.46 | + 0.07 | 0.65 | — 0.27 | 0.34 |
| 35 | 5 | Valine | 0.55 | — 0.09 | 0.68 | — 0.33 | 0.24 |
| 36 | 6 | Leucine | 0.65 | — 0.27 | 0.76 | — 0.50 | 0.23 |
| 37 | 4 | β-Amino-butyric | 0.43 | + 0.12 | 0.60 | — 0.18 | 0.30 |
| 38 | 5 | γ-Amino-valeric | 0.45 | + 0.09 | 0.62 | — 0.21 | 0.30 |
| 39 | 6 | Isoleucine | 0.64 | — 0.25 | 0.74 | — 0.45 | 0.20 |
| | *Mono-amino, mono-amide acids* | | | | | | |
| 40 | 4 | Asparagine | 0.28 | + 0.41 | 0.30 | + 0.37 | 0.04 |
| 41 | 5 | Glutamine | 0.29 | + 0.39 | 0.34 | + 0.29 | 0.10 |
| | *Mono-amino, mono-hydroxy acids* | | | | | | |
| 42 | 3 | Serine | 0.35 | + 0.27 | 0.43 | + 0.12 | 0.15 |

(*continued on p. 394*)

TABLE I (*continued*)

| *Code No.* | *Carbon No.* | *Compound* | *n-Propanol–2 N ammonia (70:30)* | | *n-Propanol–water-sat. $SO_2$ (70:30)* | | *$(R_M)$ alkaline $-(R_M)$ acid* |
|---|---|---|---|---|---|---|---|
| | | | $R_F$ | $R_M$ | $R_F$ | $R_M$ | |
| | *Di-amino acids* | | | | | | |
| 43 | 5 | Ornithine mono-HBr | 0.27 | + 0.43 | 0.28 elong. | + 0.41 | 0.02 |
| 44 | 6 | Lysine mono-HCl | 0.29 | + 0.39 | 0.30 elong. | + 0.37 | 0.02 |
| | *Other amino acids* | | | | | | |
| 45 | 5 | Proline | 0.45 | + 0.09 | 0.55 | — 0.09 | 0.18 |
| 46 | 5 | Hydroxyproline | 0.37 | + 0.23 | 0.46 | + 0.07 | 0.16 |
| 47 | 6 | Citrulline | 0.32 | + 0.33 | 0.42 | + 0.14 | 0.19 |
| 48 | 9 | Tyrosine | 0.46 | + 0.07 | 0.63 | — 0.23 | 0.30 |
| 49 | 11 | Tryptophan | 0.58 | — 0.14 | 0.68 tail | — 0.33 | 0.19 |
| 50 | 9 | Phenylalanine | 0.64 | — 0.25 | 0.71 | — 0.39 | 0.14 |
| 51 | 5 | Methionine | 0.56 | — 0.10 | 0.68 | — 0.33 | 0.23 |
| 52 | 6 | Arginine mono-HCl | 0.25 | + 0.48 | 0.36 s. tail | + 0.25 | 0.23 |
| | *Halogen-acids* | | | | | | |
| 53 | 3 | 2-Bromo-propionic | 0.63 | — 0.23 | 0.87 | — 0.83 | 0.60 |
| 54 | 4 | 2-Bromo-*n*-butyric | 0.67 | — 0.31 | 0.88 | — 0.86 | 0.55 |
| 55 | 5 | 2-Bromo-*n*-valeric | 0.73 | — 0.43 | 0.90 | — 0.95 | 0.52 |
| 56 | 6 | 2-Bromo-caproic | 0.77 | — 0.52 | 0.91 | — 1.00 | 0.48 |
| 57 | 2 | Trichloro-acetic | 0.74 | — 0.45 | 0.84 | — 0.72 | 0.27 |
| | *Mono-phenyl acids* | | | | | | |
| 58 | 7 | Benzoic | 0.64 | — 0.25 | 0.91 | — 1.00 | 0.75 |
| 59 | 8 | Phenylacetic | 0.66 | — 0.29 | 0.91 | — 1.00 | 0.71 |
| 60 | 9 | 3-Phenyl-propionic | 0.71 | — 0.39 | 0.92 | — 1.06 | 0.67 |
| 61 | 11 | 5-Phenyl-*n*-valeric | 0.76 | — 0.50 | 0.94 | — 1.19 | 0.69 |
| 62 | 9 | Phenyl-lactic | 0.68 | — 0.33 | 0.91 | — 1.00 | 0.67 |
| 63 | 9 | Cinnamic | 0.70 | — 0.37 | 0.91 | — 1.00 | 0.63 |

(*continued on p. 395*)

TABLE I (*continued*)

| Code No. | Carbon No. | Compound | *n-Propanol–2 N ammonia (70:30)* | | *n-Propanol–water-sat. SO₂ (70:30)* | | $(R_M)$ *alkaline* —$(R_M)$ *acid* |
|---|---|---|---|---|---|---|---|
| | | | $R_F$ | $R_M$ | $R_F$ | $R_M$ | |
| | | DICARBOXYLIC ACIDS | | | | | |
| | *Unsubstituted saturated acids* | | | | | | |
| 64 | 2 | Oxalic | 0.17 | +0.69 | 0.67 | −0.31 | 1.00 |
| 65 | 3 | Malonic | 0.20 | +0.60 | 0.76 | −0.50 | 1.10 |
| 66 | 4 | Succinic | 0.23 | +0.53 | 0.79 | −0.57 | 1.10 |
| 67 | 5 | Glutaric | 0.27 | +0.43 | 0.82 | −0.66 | 1.09 |
| 68 | 6 | Adipic | 0.30 | +0.37 | 0.84 | −0.72 | 1.09 |
| 69 | 7 | Pimelic | 0.35 | +0.27 | 0.87 | −0.83 | 1.10 |
| 70 | 8 | Suberic | 0.40 | +0.18 | 0.89 | −0.91 | 1.09 |
| 71 | 9 | Azelaic | 0.47 | +0.05 | 0.90 | −0.95 | 1.00 |
| 72 | 10 | Sebacic | 0.53 | −0.05 | 0.91 | −1.00 | 0.95 |
| 73 | 5 | Ethyl-malonic | 0.29 | +0.39 | 0.87 | −0.83 | 1.22 |
| 74 | 6 | 3-Methyl-glutaric | 0.31 | +0.35 | 0.86 | −0.79 | 1.14 |
| 75 | 7 | 2,2-Dimethyl-glutaric | 0.35 | +0.27 | 0.89 | −0.91 | 1.18 |
| 76 | 7 | 3,3-Dimethyl-glutaric | 0.31 | +0.35 | 0.88 | −0.86 | 1.21 |
| 77 | 8 | 3-Methyl-3-ethyl-glutaric | 0.37 | +0.23 | 0.89 | −0.91 | 1.14 |
| | *Mono-hydroxy acid* | | | | | | |
| 78 | 4 | Malic | 0.20 | +0.60 | 0.70 | −0.37 | 0.97 |
| | *Di-hydroxy acid* | | | | | | |
| 79 | 4 | Tartaric | 0.18 | +0.66 | 0.56 | −0.10 | 0.76 |
| | *Mono-amino acids* | | | | | | |
| 80 | 4 | Aspartic | 0.19 | +0.63 | 0.44 | +0.10 | 0.53 |
| 81 | 5 | Glutamic | 0.20 | +0.60 | 0.53 | −0.05 | 0.65 |
| 82 | 6 | α-Amino-adipic | 0.21 | +0.57 | 0.57 | −0.12 | 0.69 |
| 83 | 7 | α-Amino-pimelic | 0.24 | +0.50 | 0.63 | −0.23 | 0.73 |

(*continued on p. 396*)

TABLE I (*continued*)

| Code No. | Carbon No. | Compound | *n-Propanol–2 N ammonia* (70:30) | | *n-Propanol–water-sat.* $SO_2$ (70:30) | | $(R_M)$ *alkaline* —$(R_M)$ *acid* |
|---|---|---|---|---|---|---|---|
| | | | $R_F$ | $R_M$ | $R_F$ | $R_M$ | |
| | *Aromatic acids* | | | | | | |
| 84 | | Phenylsuccinic | 0.39 | + 0.19 | 0.86 | — 0.79 | 0.98 |
| 85 | | Phthalic | 0.33 | + 0.31 | 0.86 | — 0.79 | 1.10 |
| | *Unsubstituted double bonded acids* | | | | | | |
| 86 | 4 | Fumaric | 0.27 | + 0.43 | 0.85 | — 0.75 | 1.18 |
| 87 | 4 | Maleic | 0.25 | + 0.48 | 0.78 | — 0.55 | 1.03 |
| 88 | 5 | Mesaconic | 0.27 | + 0.43 | 0.85 | — 0.75 | 1.18 |
| 89 | 5 | Citraconic | 0.28 | + 0.41 | 0.84 | — 0.72 | 1.13 |
| 90 | 5 | Itaconic | 0.29 | + 0.39 | 0.84 | — 0.72 | 1.11 |
| | | | TRICARBOXYLIC ACIDS | | | | |
| | *Unsubstituted saturated acids* | | | | | | |
| 91 | 6 | Tricarballylic | 0.13 | + 0.83 | 0.80 | — 0.60 | 1.43 |
| | *Hydroxy acids* | | | | | | |
| 92 | 6 | Citric | 0.11 | + 0.91 | 0.69 | — 0.35 | 1.26 |
| 93 | 6 | Isocitric | 0.11 | + 0.91 | 0.69 | — 0.35 | 1.26 |
| | *Aromatic acid* | | | | | | |
| 94 | 9 | Trimesic | 0.12 | + 0.86 | 0.87 | — 0.83 | 1.69 |
| | *Unsubstituted double bonded acids* | | | | | | |
| 95 | 6 | *cis*-Aconitic | 0.14 | + 0.79 | 0.74 | — 0.45 | 1.24 |
| 96 | 6 | *trans*-Aconitic | 0.14 | + 0.79 | 0.86 | — 0.79 | 1.58 |

(*continued on p. 397*)

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TABLE I (*continued*)

| Code No. | Carbon No. | Compound | *n-Propanol–2 N ammonia (70: 30)* | | *n-Propanol–water-sat. $SO_2$ (70: 30)* | | $(R_M)$ *alkaline* —$(R_M)$ *acid* |
|---|---|---|---|---|---|---|---|
| | | | $R_F$ | $R_M$ | $R_F$ | $R_M$ | |
| | | | TETRACARBOXYLIC ACIDS | | | | |
| 97 | 10 | Prehnitic | 0.04 | + 1.38 | 0.77 | — 0.52 | 1.90 |
| 98 | 10 | Pyromellitic | 0.05 | + 1.28 | 0.81 | — 0.63 | 1.91 |
| | | | INORGANIC ACIDS | | | | |
| 99 | | Orthophosphoric | 0.11 | + 0.91 | 0.62 | — 0.21 | 1.12 |
| 100 | | Sulphuric | 0.18 | + 0.66 | 0.31 | + 0.35 | 0.31 |
| 101 | | Hydrochloric | 0.43 | + 0.12 | 0.51 | — 0.02 | 0.14 |
| 102 | | Nitric | 0.53 | — 0.05 | 0.61 | — 0.19 | 0.14 |
| 103 | | Perchloric | 0.62 | — 0.21 | 0.71 | — 0.39 | 0.18 |
| | | | NEUTRAL COMPOUNDS | | | | |
| 104 | 18 | Raffinose | 0.20 | + 0.60 | 0.20 | + 0.60 | 0.00 |
| 105 | 12 | Sucrose | 0.35 | + 0.27 | 0.36 | + 0.25 | 0.02 |
| 106 | 6 | Glucose | 0.40 | + 0.18 | 0.41 | + 0.16 | 0.02 |
| 107 | 6 | Sorbitol | 0.41 | + 0.16 | 0.42 | + 0.14 | 0.02 |
| 108 | 6 | Dulcitol | 0.43 | + 0.12 | 0.43 | + 0.12 | 0.00 |
| 109 | 6 | Fructose | 0.43 | + 0.12 | 0.45 | + 0.09 | 0.03 |
| 110 | 5 | Ribose | 0.50 | 0.00 | 0.50 s. tail | 0.00 | 0.00 |
| 111 | 3 | Glycerol | 0.59 | — 0.16 | 0.60 | — 0.18 | 0.02 |

elong. = elongated spot; s. tail = short tail.

alcohol that could be used although its resolving power for closely similar acids was not as great as the higher alcohols.

The investigation also included a search for a solvent that would give a constant value of $\Delta R_M$ for the addition of $-CH_2-$ in a few of the homologous series of acids. This value was not always constant for all the series of acids examined in any one alcohol but here again the two propanol solvents chosen were one of the best combinations.

(c) Acids and bases added to the solvent must be volatile to allow for the subsequent operation of a pH indicator spray and they both must be strong enough to suppress the ionisation of the acidic or basic groups in the acid to be investigated.

Ammonia is a strong enough base to form salts with all the organic acids studied here and for many of the ampholytic amino acids but may not be strong enough to suppress the basic groups in amino acids such as arginine. However its volatility and purity made it superior to other bases. Paper chromatograms developed in alcohol–ammonia mixtures and then sprayed with Universal Indicator or silver nitrate always show a false front whose position is dependent on the nature of the alcohol and the amount of water (and/or ammonia) in the solvent. With the solvent described above, this front appeared at an $R_F$ of approx. 0.40 as a line of demarcation behind which the paper background was faintly alkaline and beyond which it was slightly acid.

On the acidic side sulphurous acid was preferred to the more commonly used formic or acetic because of its greater strength (*cf.* VAS[9]). This is particularly necessary when dealing with a strong organic acid, such as oxalic acid, in the form of its ammonium salt as the method described depends largely on the fact that the acid was forced completely into the unionised state. The ammonium ion (from the ammonium salts of the acids applied to the starting line) interacts with sulphur dioxide in the solvent and results in an extra acid spot which has an $R_F$ value of 0.46. This should not cause confusion with an unknown acid spot because it occurred in a part of the chromatogram away from all other "indicator-positive" organic acids studied, it was also slower in colouring up and less intense than the free acid spots.

A further advantage of sulphur dioxide is its volatility which allows the paper to be sprayed as soon as it is dry without any further treatment. This makes it possible to chromatograph semi-volatile acids which would otherwise be lost in the steaming, heating or leaving overnight required in the removal of formic or acetic acids from the paper.

### *The effect of the carboxyl group on the $R_F$ value* (Fig. 1)

The dominating effect of the carboxyl group on the $R_F$ values of the compounds is illustrated in Fig. 1 which contains all the results of Table I in the form of a two-dimensional graph of $R_F$ values for the two solvents. Arbitrary boundaries have been drawn to separate the acids according to their carboxyl number in such a way that each acid of Table I falls into its correct grouping.

Neutral compounds, *i.e.* those that contain no ionisable groups and which are chemically unaffected by the presence of ammonia or sulphur dioxide, have closely similar $R_F$ values in the two solvents. Ideally the $R_F$ should be identical and the values irrespective of constitution should fall on a 45° slope line. In practice it was found that the values in the acid solvent were equal to or slightly higher than the alkaline solvent. There was no visible evidence on the chromatograms of inter-action between the sugars and sulphur dioxide, nor of hydrolysis of the polysaccharides.

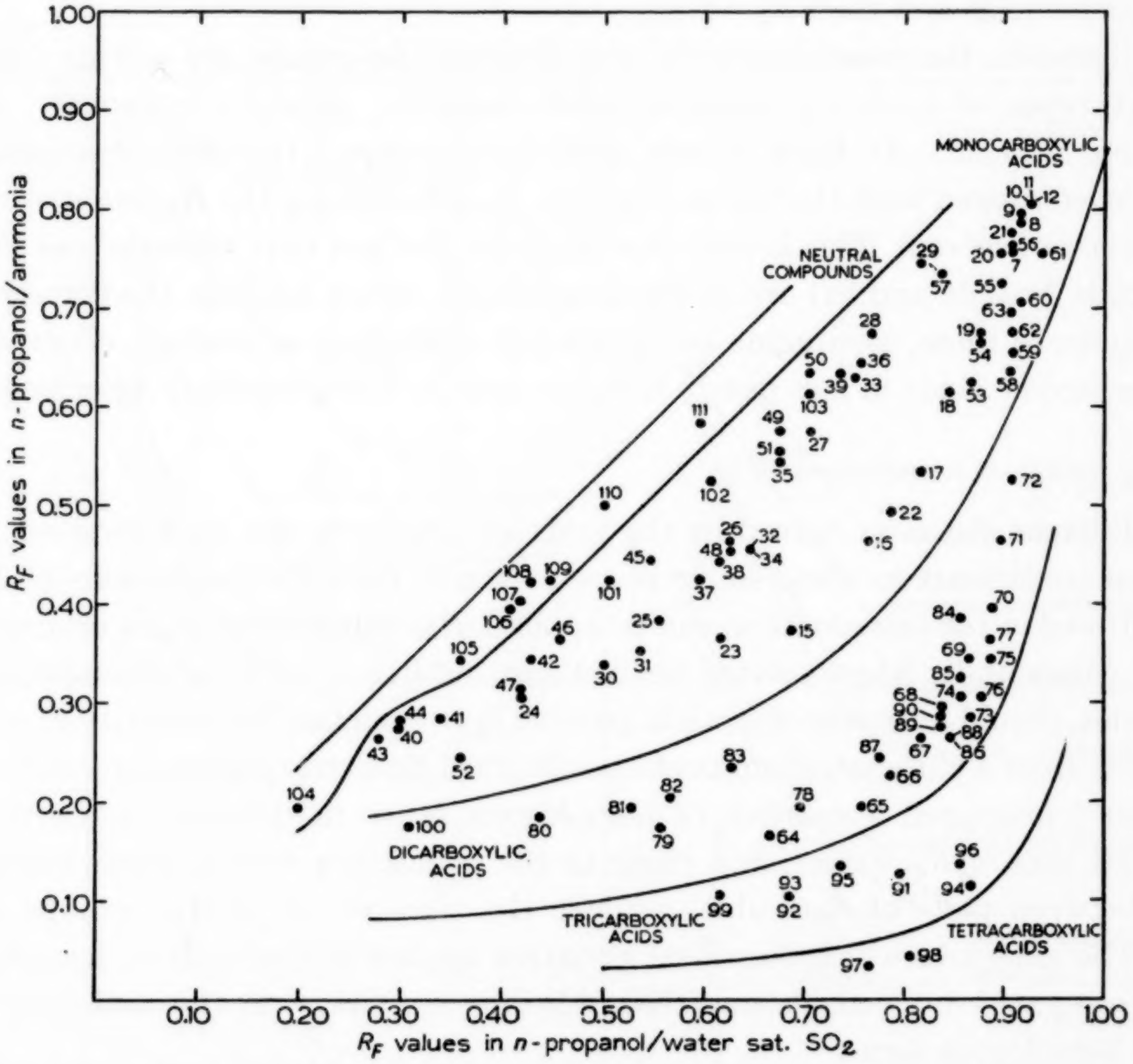

Fig. 1. $R_F$ values in n-propanol–ammonia plotted against $R_F$ values in n-propanol–water saturated with $SO_2$, for compounds listed in Table I.

The separation (in Fig. 1) between the slower running neutral compounds raffinose and sucrose (code Nos. 104 and 105) and the monocarboxylic amino aicds, asparagine, ornithine and lysine (code Nos. 40, 43 and 44) is achieved only by drawing an irregular boundary between them. This would be impossible if other neutral compounds, with $R_F$ values intermediate between raffinose and sucrose, had been included. As the neutral compounds and the amino acids were identified by different sprays there was no difficulty in placing each in its proper group.

A few inorganic acids are also included in Fig. 1. The strong, monobasic, mineral acids—hydrochloric, nitric and perchloric (code Nos. 101, 102 and 103)—are in the monocarboxylic group whereas dibasic sulphuric acid (code No. 100) is just in the dicarboxylic group. This is an unexpected agreement between the behaviour of the weak organic acids and the strong mineral acids because the sulphurous acid in the solvent is presumably not strong enough to affect the ionisation of the latter. On the other hand, tribasic orthophosphoric acid (code No. 99) is a much weaker acid and therefore there is some justification for its appearance along with the other tricarboxylic acids. A further investigation of inorganic acids was not attempted, so it is not possible to say, at this stage, whether there is any direct relationship between the basicity of inorganic acids and their $R_F$ values.

Included in the monocarboxylic and dicarboxylic groups are a wide variety of different types of acids *e.g.* unsubstituted, halogen-, phenyl-, hydroxyl-, amino-, double bonded acids, etc. Each of these substituted groups is therefore of secondary importance, compared with the carboxyl group, in determining the $R_F$ value of the acid in this class of solvent. This is well illustrated by the fact that aspartic and glutamic acids (code Nos. 80 and 81) are in the dicarboxylic group whereas the corresponding monoamides of these, asparagine and glutamine (code Nos. 40 and 41), occur with the monocarboxylic acids in fact nearly with the neutral compounds as described above.

### *The $R_M$ values of homologues* (Fig. 2)

If the different distances moved by the acids are primarily due to differences in their partition coefficients in these single phase solvents, then the predictions of MARTIN etc., outlined in the introduction, can be applied. $R_M$ values from series of structurally related compounds, when plotted against unit additions of a substituted group in each series, should form sets of straight parallel lines such that the identity of unknown acid spots from a chromatogram could be obtained by extrapolation or interpolation. LEDERER[10] examined a number of homologous series in different solvents, from published lists of $R_F$ values, and came to the conclusion that a linear relationship exists between plots of $R_M$ values against the number of $-CH_2-$ groups in each series. The extent to which this ideal situation applies to the system described here was investigated by considering the addition of $-CH_2-$ to the molecule in nine similar homologous series.

The $R_M$ values for these homologues are plotted against carbon number in Figs. 2A and 2B for the two solvents. These plots show that the introduction of -phenyl, -Br, and $-CH_2-$ into the molecule results in a decrease of $R_M$ value (increase in $R_F$) whereas -OH, $-NH_2$, and -COOH increase the $R_M$ value in both solvents. The values of the phenyl-substituted fatty acids (code Nos. 58–61, Table I) are plotted in these two figures against the number of carbon atoms in the fatty acid part of the molecule alone. Calculations on the $R_F$ values of HASHMI AND CULLIS[5] show that the substitution of iodine into the fatty acid molecule also reduces the $R_M$ value of the acid in a very similar propanol–ammonia solvent. The extent of the reduction is slightly greater than that resulting from the corresponding introduction of bromine into the molecule.

In both solvents the majority of series show a close approximation to linearity up to compounds containing 8 carbon atoms; above this value the straight-chain and hydroxy-monocarboxylic acids rapidly approach a limiting value as found by ISHERWOOD AND HANES[4] in similar alkaline solvents. Although straight lines or regular curves have been drawn to represent the behaviour of the series, the individual members often show small departures from the line. By always comparing all members of any one series on the same paper, at the same time, these minor variations have been shown to be repeatable and in most cases are true expressions of the individuality of the separate acids. A good example of this is often shown by the first member of a homologous series.

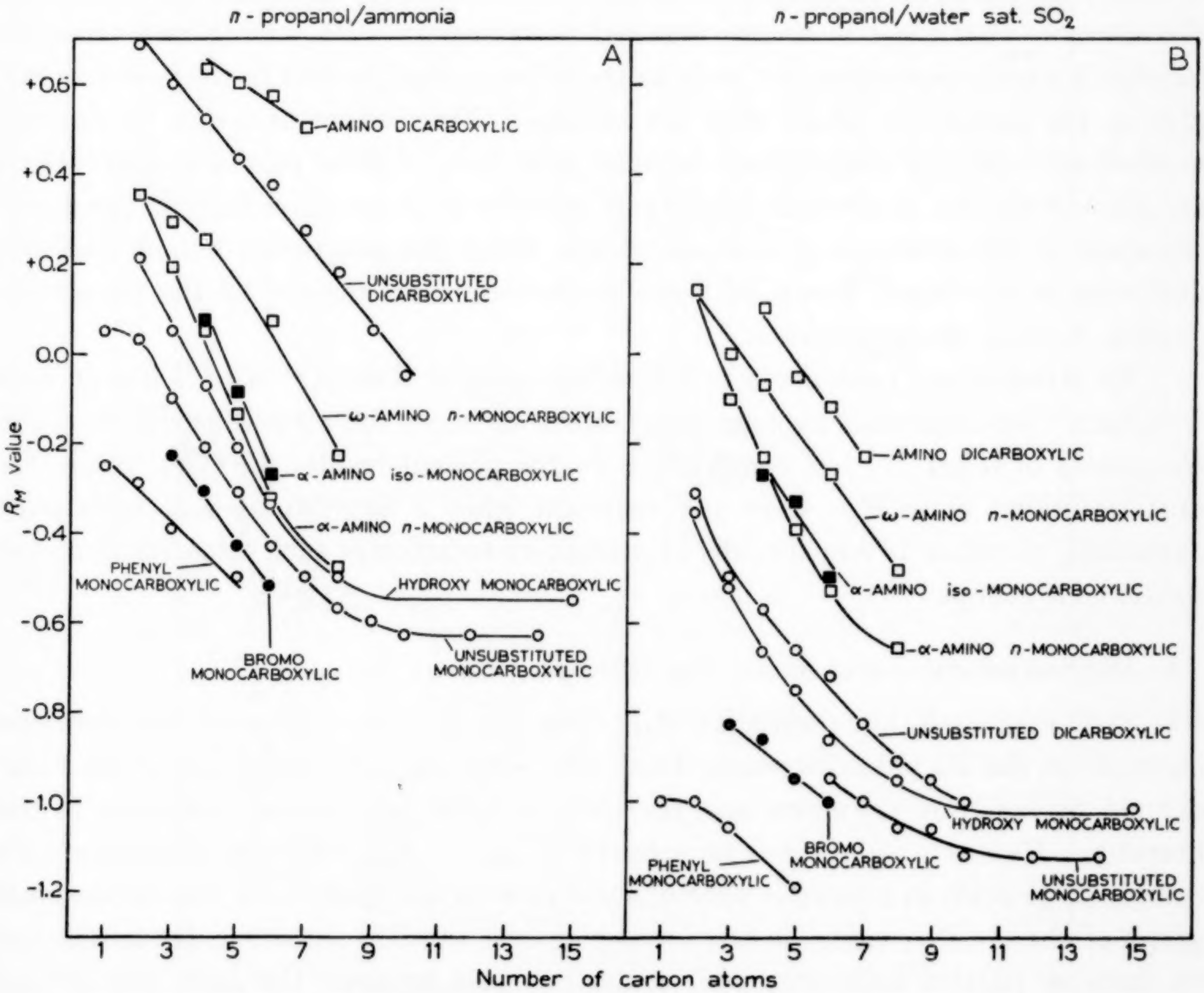

Fig. 2. Variation of $R_M$ values with number of carbon atoms in homologous series. □ ■ amino acids, ○ ● non-amino acids.

The lines representing the series show a general similarity of slope but they are not strictly parallel to one another. In fact the divergencies from parallel are such that it is not possible to give a precise $\Delta R_M$ value for the addition of $-CH_2-$ in all the series; even over the linear parts of the graph the $\Delta R_M$ values of different lines vary, from 0.05 to 0.15 approximately, in both solvents.

The short homologous series of α-amino-iso-acids are very similar to the α-amino-*n*-acids a condition which corresponds to the lack of distinction of *n*- from iso- in the non-substituted acids butyric and valeric. On the other hand, as noted by SCHAUER AND BULIRSCH[11], the position of the amino group in the molecule has a relatively large influence on the final $R_M$ value of the compound. An acid containing a terminal

($\omega$-) amino group has a higher $R_M$ value (lower $R_F$) than the corresponding acid with an $\alpha$-amino group. The two amino acids containing $-NH_2$ substituted part way down the chain (*i.e.* $\beta$-amino-*n*-butyric and $\gamma$-amino-*n*-valeric) adopt intermediate positions between these two extremes.

At least part of the explanation of these divergencies must lie in the assumptions made by MARTIN which were to a first approximation only. It must be assumed therefore that the partition coefficients of isomers are of the same order of magnitude only. Furthermore that the free energy required to transport $-CH_2-$ from one phase to another is partly dependent not only on the other groups present in the molecule but also on the manner in which they are arranged. This cannot of course be directly verified with the two single phase solvents used here. If these practical reservations are allowed for, the theoretical predictions prove to be of immense value in the determination of the structure of unknown acids. From the practical point of view the difference in $R_F$ values shown by some isomers increases the use of the chromatographic method of identification.

No attempt has been made to follow the work of REICHL[12] or of SCHAUER AND BULIRSCH[11] who calculate average $\Delta R_M$ values for many substituent groups from the $R_M$ values of relatively few compounds. In the present results the $\Delta R_M$ values for unit additions of $-CH_2-$ were not constant when a large number of acids were examined, therefore it was considered premature to attempt such calculations on the substituent groups.

### *The alkaline solvent—acid solvent $R_M$ difference* (Fig. 3)

REICHL[13] expressed his results as $R_M = \log [R_F/(1-R_F)]$ because this function increases as the $R_F$ value increases. This value is the negative logarithm of the value defined by equation (2) above and therefore to avoid any further confusion in the literature, REICHL's values will be referred to as $(-R_M)$ values in this paper. He examined 36 acids in 2 pairs of solvents and plotted his results as a two-dimensional graph of the $(-R_M)$ values in one solvent against those in the other. He found that on drawing parallel lines across his graph he could separate the acids into groups according to the number of -COOH groups they contain. In one set of solvents the amino acids occurred in the wrong group and in both sets of solvents phthalic and maleic acids were anomalous, which he concluded was due to interaction between the adjacent carboxyl groups—an "*ortho* effect" (*cf.* BAKER[14], BATE-SMITH AND WESTALL[3]). In the present paper, no such anomalous behaviour was noticeable with these or other acids containing adjacent -COOH groups.

REICHL also noted that the difference in the $(-R_M)$ values for each acid, between an acidic and a neutral solvent, was a good index of the number of carboxyl groups in that acid. A similar $R_M$ difference has been used by MACEK AND VEJDĚLEK[15] to determine the number of $\alpha$-glycol groups in the veratrum alkaloids. In their method the $R_M$ difference is between the values obtained for each alkaloid, chromatographed first on an ordinary paper and then, under similar conditions, on a paper impregnated with boric acid.

Values of the expression ($R_M$) alkaline solvent—($R_M$) acid solvent are tabulated in Table I. With the larger number of acids considered here the range of values included in the monocarboxylic group (0.02 to 0.75) overlap somewhat the range covered by the dicarboxylic group (0.53 to 1.22). However, to illustrate the value of this expression, the homologous series and other compounds are graphed in Fig. 3 with the $R_M$

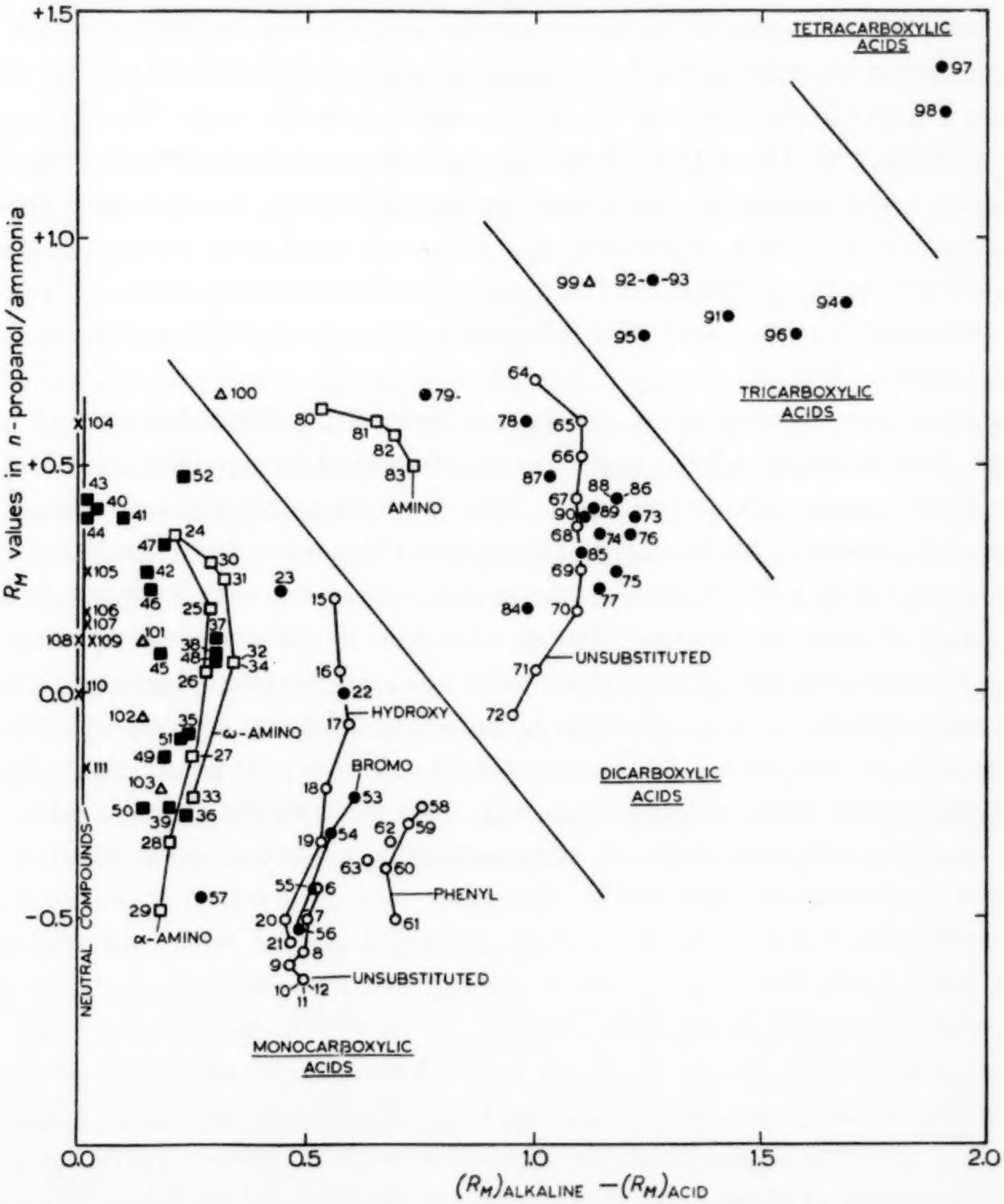

Fig. 3. $R_M$ values in $n$-propanol–ammonia of compounds listed in Table I, plotted against the difference in $R_M$ values between the two solvents. ×, neutral compounds; △, inorganic acids; □ ■, amino organic acids; ○ ●, non-amino organic acids.

difference between the two solvents as abscissa against the $R_M$ in propanol–ammonia as ordinate. Plotted in this way the overlapping of values from one carboxyl group to another is avoided and it is possible to draw an equi-spaced series of parallel lines to separate the acids into their correct groups.

Neutral compounds occur on the extreme left of the graph and are represented by the vertical line at 0.01 $R_M$ difference between the two solvents. The same overlapping with a few of the amino acids, as previously explained for Fig. 1, is also noticeable here. The homologous series are shown connected point to point by full lines which are nearly vertical and the points composing them are in order of increasing carbon number downwards. A vertical line for these series would mean that the addition of $-CH_2-$ to an acid produced the same change in partition coefficient irrespective of whether the acid was ionised or unionised.

The homologous series of monocarboxylic $\alpha$-amino-iso-acids (code Nos. 34–36) are not connected by lines in the figure because they would obscure part of the series of $\alpha$-amino-*n*-acids (code Nos. 24–29) and $\omega$-amino-*n*-acids (code Nos. 30–33) which are shown connected. These three homologous series together with $\beta$-aminobutyric, $\gamma$-aminovaleric and isoleucine (code Nos. 37–39) all occupy a very small sub-area of the monocarboxylic group. Therefore any unknown acid spot which occurs in this sub-area is likely to be an isomer of the amino-substituted fatty acids. Tyrosine (code No. 48), an aromatic amino acid, also falls inside this area and is therefore an exception to the statement.

Similar sub-groups and areas can be marked off for other derivatives and configurations. For example, all the amino acids of Table I occupy the left hand area of their respective carboxylic groupings in Fig. 3, whereas all the non-amino organic acids are quite separate to the right. The intervening space between these two sub-groups will probably be occupied by the mono-, di- or tri- etc. hydroxy-substituted organic acids. A start in this direction can be seen in the sequence succinic–malic–tartaric acid (code Nos. 66, 78, 79) which have 0, 1 and 2 hydroxyl groups respectively with the same number of carbon atoms in each. The addition of each hydroxyl group brings the acid position nearer to the amino acid sub-group. It is anticipated therefore that certain heavily hydroxylated acids will occur in with the amino acids.

The three homologous series of monocarboxylic acids *i.e.* unsubstituted, bromo- and hydroxy-substituted respectively, also occur very close together. But the homologous phenyl-substituted acids (code Nos. 58–61) together with phenyl-lactic and cinnamic acids (code Nos. 62, 63) are to the right of these and suggest the presence of a separate aromatic sub-group. Finally, it is worth mentioning that the few inorganic acids studied occupy positions to the left of each carboxylic group.

Recently it has been found that certain substituted aromatic acids such as syringic and protocatechuic acids do not fit into their correct carboxyl grouping. Detailed discussion of these and other inter-relationships contained in this paper will be postponed until more information concerning related compounds has been obtained.

## CONCLUSION

As there is such a close and definite relationship between $R_M$ value and molecular structure of an acid it is clear that there will be many more acids than those already studied which will fit into the carboxyl group areas drawn here.

The scheme outlined should prove useful not only in conjunction with selective spray reagents but also in the examination of unknown compounds detected by non-specific reagents. For example, with radioactive isotopes revealed by autoradiographs it may be possible to show by this method

(a) whether the compound is neutral or not;
(b) the number of carboxyl groups in the molecule;
(c) the presence of $-NH_2$ groups;
(d) an indication of other substituted groups such as phenyl or hydroxyl.

## ACKNOWLEDGEMENTS

I would like to thank Dr. E. C. Bate-Smith, Dr. M. Ingram, Dr. H. G. Wager and Mr. F. A. E. Porter for the help and guidance they have given throughout this investigation.

## SUMMARY

An acidic and a basic solvent containing the same proportions of propanol and water have been used for the study of about 100 organic acids by paper chromatography. The acid and the base were solutions of the gases sulphur dioxide or ammonia in water and therefore were readily removed after chromatography. Sulphurous acid has the advantage of being a stronger acid than those normally used, *e.g.* formic and acetic acid. The $R_M$ values of the organic acids in the two solvents can be arranged into clear-cut groups dependent on the number of carboxyl and other substituted groups present, in such a way that the number of carboxyl groups in an unknown acid can be confidently predicted. An indication can also be obtained of other groups such as alkyl, aryl, amino, hydroxy, bromo etc. which may be present, and the manner in which they are arranged. The results include nine homologous series whose members differ from one another only in the additon of $-CH_2-$ to the molecule.

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# A TESLA DISCHARGE DETECTOR FOR GAS CHROMATOGRAPHY[*]

JAMES C. STERNBERG[**] AND RICHARD E. POULSON[**]
*Kedzie Chemical Laboratory, Michigan State University, East Lansing, Mich. (U.S.A.)*

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During the course of a spectroscopic study of glow discharges in gases excited by a high frequency Tesla coil source (an ordinary laboratory leak-tester), it became apparent that the properties of the electrodeless Tesla discharge were a very sensitive function of the composition of the gas. A discharge in a carrier gas is visibly altered by small amounts of added vapor. Two new detectors for gas chromatography based upon the properties of such discharges have been developed and offer certain unique advantages over the other means of detection; their greatest advantage is probably in the simplicity and economy of construction of a high sensitivity detector and its associated electrical measuring circuit.

The new detectors utilize measurement of:

(a) the emitted light intensity, and

(b) a direct current signal produced by inserting a pair of probe electrodes asymmetrically in the discharge.

Both detectors can be constructed without elaborate fabrication and with a minimal expenditure for components. Both means of detection can, if desired, be incorporated into a single detector unit without complication; a detector designed for the current measurement can also be used conveniently for measurements of light intensity.

## APPARATUS

The discharge tube portion of the detector is simply a glass capillary tube (0.5–2 mm diameter) attached directly to the column exit. A laboratory Tesla coil leak-tester provides the source of excitation of the discharge, which is transmitted through a piece of aluminum foil connected to the high voltage probe terminal (the probe itself is removed and the aluminum foil inserted in the end of the leak-tester in place of the probe) and wrapped around the capillary tubing. A small diameter wire lodged in the capillary and extending downstream from the region encircled by the aluminum foil serves as a low work function source of electrons and stabilizes the discharge, which otherwise would have to depend for initiation upon more difficult removal of electrons from the glass walls.

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[*] Part of a thesis to be submitted by Mr. POULSON to the Graduate School, Michigan State University, for the degree of Doctor of Philosophy.

[**] At Corporate Research Department, Beckman Instruments, Fullerton, California.

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The discharge occurs downstream from the end of the inserted wire to a region nearer ground potential. A similar "electrodeless electrode" can serve as the ground, by inserting another free piece of wire in the capillary and surrounding the region of the capillary containing the wire by a grounded piece of aluminum foil. A better ground can be prepared by attaching the capillary to a grounded metal tubing connector, using "O"-ring seals. For the direct current measurement, a brass "T"-tube connector can conveniently be used. The discharge capillary tube is connected to one arm of the "T"-tube; an inner electrode is mounted in glass and connected to the opposite arm of the "T"-tube, with the inner electrode extending within the "T"-tube very nearly to the end of the discharge capillary tube; the third arm of the "T"-tube contains the exit tube, which may be vented to the atmosphere or may lead to a manometer, flow control valve, ballast flask, and vacuum pump or water aspirator. The inner electrode is connected to ground, and the body of the "T"-tube serves as the other electrode for the direct current measurement.

## PROPERTIES OF THE DISCHARGE

The Tesla coil sets up a high voltage (about 50 kV), high frequency (about 3 megacycles) electrical field, which initiates and maintains the discharge. A visible glow discharge occurs in air, nitrogen, helium, or argon carriers at reduced pressures, and extends to more than one atmosphere pressure in helium or argon and to about 350 mm in nitrogen and in air. The discharge is visibly altered by even very small quantities of sample gases (organic compounds are particularly characterized by a bright blue-green color, predominantly due to bands of $C_2$). The visible indication itself provides an excellent qualitative identification of sample bands for lecture demonstrations of gas chromatography or for monitoring preparative column separations in the organic laboratory.

A photoconducting cell (cadmium sulfide or cadmium selenide) mounted on the side of the capillary discharge tube gives convenient and sensitive quantitative light intensity measurement; the circuitry required is a battery-supplied direct current circuit in which the current can be measured directly or the voltage drop across a selected resistor can be observed, using either a dial instrument or a recording potentiometer. The use of filters or a monochromator with the photocell makes possible preferential observation of emission due to the carrier gas or emission due to sample gas fragments (such as $C_2$, which is generally observed for organic molecules); with observation of carrier gas emission, the quenching by samples is detected, while in observation of sample emission an enhancement of emission indicates presence of a sample in the carrier. Particularly high sensitivity has been observed with argon carrier in a detector at atmospheric pressure. The use of a spectrophotometer equipped with photomultiplier, or of photographic observation of spectra, offers a possible qualitative identification of certain sample components.

The electrical properties of the discharge afford another means of detection. The brass "T"-tube with its inner electrode actually serves as a rectifier of the radiofrequency Tesla discharge, so that a direct current signal is obtained without an applied voltage; KARMEN AND BOWMAN[1] have reported a similar effect in their radiofrequency discharge detector, which appears to involve very similar operating principles despite their very different experimental arrangement. There are other reports in the literature of rectification from radiofrequency[2] and Tesla-spark discharges[3]. The signal depends upon the asymmetrical location of the electrodes, and must be attributed to preferential capture of electrons at the more accessible center electrode, with the positive ions becoming discharged at the outer jacket. The inner electrode is thus negative and the jacket is positive with respect to the external circuit. The circuit used includes a bias battery for adjusting the zero level, a resistance-capacitance network for signal and noise attenuation, and an indicating or recording microammeter or millivoltmeter.

## RESULTS

With the direct-current measurement, the detector seems to be most applicable at reduced pressures. It is then possible to use essentially any desired carrier gas and to obtain a signal for any sample gas in that carrier—there are no limitations due to ionization potentials or other properties of the gases. Responses to sample gases in a given carrier may be to produce enhancement of current or to produce a quenching of current; both types of response are illustrated in Fig. 1, for methane and helium, respectively, in air carrier at 3.6 mm. Both response for the pure carrier and sensitivity to samples show a pressure dependence and pass through a maximum at some particular pressure; these properties are illustrated in Fig. 2, again for air carrier.

As in other ionization detectors[4-6], sensitivity is characteristic of each particular sample gas, in addition to its dependence on carrier gas, pressure, and detector geometry. An approximate comparison of sensitivities for different sample gases has been obtained by comparing responses to large injections of different sample gases in

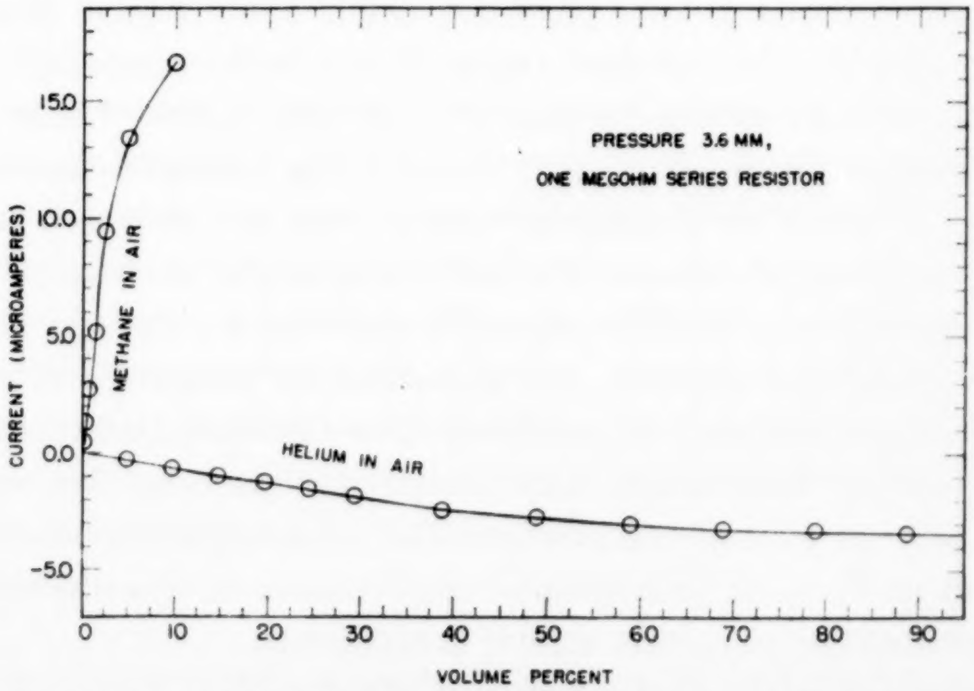

Fig. 1. Methane solutions in air and helium solutions in air; air carrier. Response relative to air *vs.* concentration.

solution in the carrier gas at a concentration of 1 % by volume, which is nearly in the linear response range (see Fig. 1); the response (sample current minus pure carrier current) is given in Table I for 1 % solutions of several different sample gases in air carrier at 3.5 mm. It is seen that response increases with number of carbon atoms within homologous series of compounds. The best sensitivity seems to be possible with argon as the carrier gas, at reduced pressure, since argon gives a discharge with exceptional stability and low noise level. Under these conditions, the Tesla discharge detector appears to be as sensitive as other types of ionization detectors, and to be unrestricted in the types of vapors detectable. However, with argon

TABLE I

RELATIVE RESPONSE IN AIR CARRIER TO 1 VOL. % SOLUTIONS IN AIR FOR VARIOUS SAMPLE GASES

Pressure = 3.7 mm, 1 MΩ series resistor

| | *Response (μA)* |
|---|---|
| Hydrogen | 2.8 |
| Acetylene | 3.6 |
| Methane | 4.7 |
| Ethylene | 5.5 |
| Ethane | 7.3 |
| Cyclopropane | 9.2 |
| Propylene | 11.4 |
| Propane | 11.6 |
| 1-Butene | 13.0 |

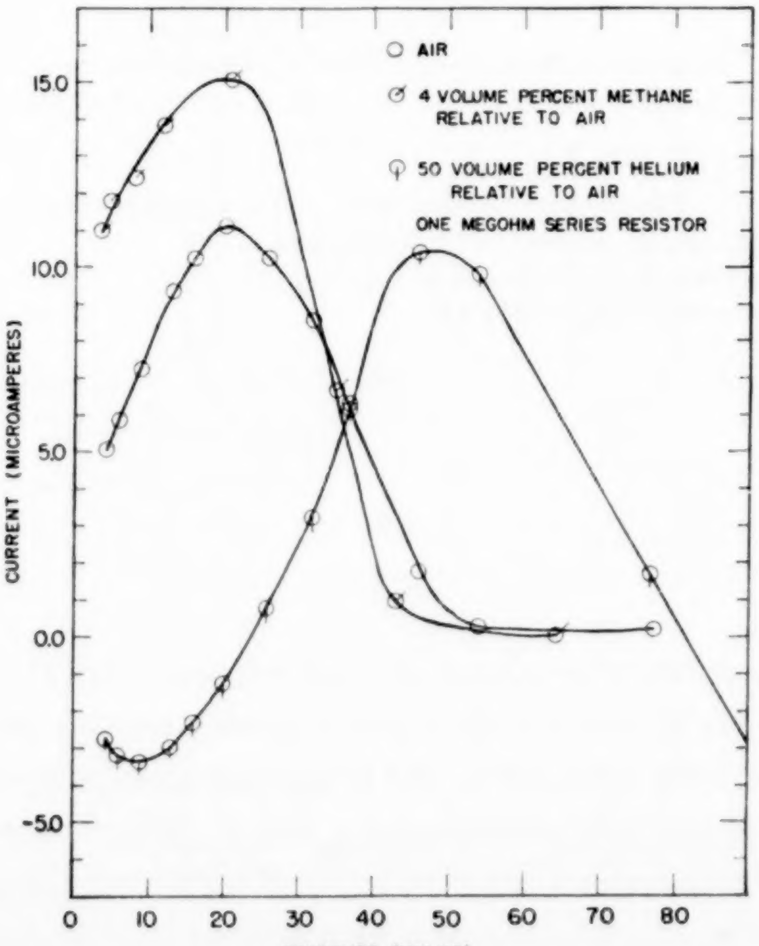

Fig. 2. Sensitivity *vs.* pressure for air, methane in air and helium in air.

carrier it has been found desirable to introduce a controlled slow flow of oxygen gas through a "T"-tube into the carrier-sample stream just before the point of excitation of the discharge. The oxygen removes free radical fragments and prevents tailing and faulty band shapes resulting from contamination of the discharge tube and electrodes with carbonaceous breakdown products.

## ACKNOWLEDGEMENT

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## SUMMARY

Two new detectors for gas chromatography, based on properties of the Tesla discharge, have been developed. Their simplicity and economy of construction suggests their use in lecture demonstrations, undergraduate laboratory instruction, and in monitoring preparative column work at the laboratory bench. The possibility of use of air as the carrier gas opens up interesting applications of the detector, with or without a column, in measurement of humidity, vapor pressures above solvents or solutions, molecular weight determinations, and observation of various atmospheric contaminants. The visible discharge can be monitored spectroscopically to obtain some information on the nature of the components.

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# PTFE-GLASS PUMP. CHROMATOGRAPHIC APPLICATIONS

A. C. ARCUS

*Nutrition Research Department, Medical School, Dunedin (New Zealand)*

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The supply of solvent under pressure to chromatographic columns is usually achieved by means of gas pressure, by using a pump, or by having a solvent reservoir at a height above the column. Gas pressure has the disadvantage that dissolved gas usually bubbles out of solution near the bottom of the column, while a high reservoir is awkward to set up and the pressure obtainable is usually limited by the height of the room. These difficulties are avoided by the use of a pump, because there is no need to have gas in contact with the solvent, and high pressures can be got easily.

This note describes a pump with a glass cylinder and a polytetrafluoroethylene (PTFE) piston, which has several useful applications in chromatography. Being made entirely of glass and PTFE it can be used with any solvent likely to be encountered. Further, a sealing rim on the piston ensures that there is no leak of solvent back past the piston, as there is with pumps of the commonly used syringe type.

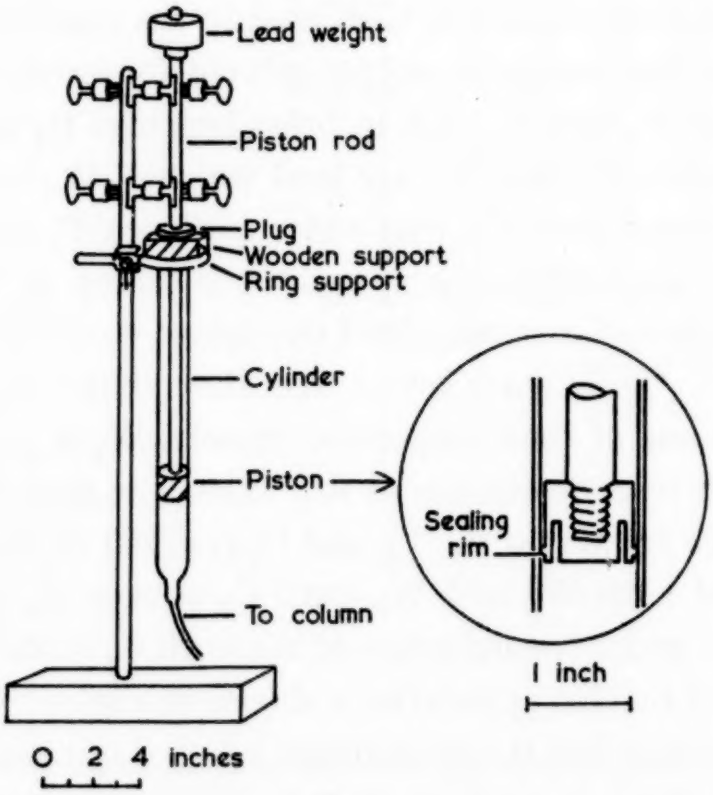

Fig. 1.

The pump is shown in Fig. 1, the inset showing the construction of the piston, which is made on a lathe from PTFE rod. The sealing rim on the piston, which has an outside diameter of 2.54 cm, is turned with a tool that has a straight edge containing a semi-circular notch 0.05 cm deep.

A piston made from polyethylene instead of PTFE works just as well, but cannot be used with non-polar solvents such as hydrocarbons because these swell the polyethylene.

The cylinder is made of 2.5 cm constant-bore borosilicate glass tubing[1,*] with a heavy rim at the top resting on the wooden support. The piston rod slides through the polyethylene plug, which fits loosely into the top of the barrel.

A weight of 1.2 lb. on the piston is just sufficient to overcome its friction in the cylinder when lubricated with solvent and a weight of 9 lb. on the piston rod gives a measured pressure of 13 lb. per square inch at the outlet. Leakage of solvent back past the piston is nil with all pressures tried (0 to 15 lb.).

As well as for supplying pressure, this pump can be used for degassing solvents in the cold as follows: It is removed from the retort stand, half filled with the solvent, and all air pushed out through the outlet, which is then plugged; the piston is then pulled out a few inches to form a vacuum over the solvent causing dissolved gas to bubble out. The process is repeated until no further gas is evolved. Even under these conditions little or no air passes the sealing rim of the piston.

The flexible capillary tubing used in this work is polyethylene cannula of 1.0 mm bore. Leak-free unions with glass capillary that do not come apart under considerable pressure are made by plugging the cannula tightly into the tapered end of the capillary.

The principle described above has been used in the construction of an apparatus shown in Fig. 2, using two pumps to supply solvent for gradient elution chromatography. The piston rods $P_1$ and $P_2$ slide in brass bearings $B_1$ and $B_2$ screwed to the upright of a retort stand. $W_1$ and $W_2$ are lead weights, $W_2$ being heavier than $W_1$. $B_3$ is a brass arm, screwed onto $P_2$, that slides easily on $P_1$ and the upright.

In one particular application the apparatus is set up as follows: The reservoir contains 100 ml of degassed methanol and the mixer 50 ml of 60% (v/v) methanol in water. The collars $C_1$ and $C_2$ are set so that they strike $B_2$ when the pistons are 1.5 cm from the bottoms of their respective vessels. $W_2$ is 4½ lb. and $W_1$ is 3 lb. $B_3$ is set so that it just rests on the top of $W_1$. When the flow of solvent is started by releasing the outer two screws on $B_2$, $P_1$ and $P_2$ coupled by $B_3$ move down together under the influence of both $W_1$ and $W_2$ until $C_2$ strikes $B_2$ when $P_2$ stops and $P_1$ continues to fall alone under the influence of $W_1$ until $C_1$ strikes $B_2$. The composition of the solvent supplied by this apparatus is shown in Fig. 3.

This sort of apparatus has the advantages pointed out previously for the pump, *viz.* that no gas need come in contact with the solvent and a high reservoir is not needed. It has the further advantage over a gravity controlled apparatus[2] in that the relative liquid heights are not determined by their densities but can be chosen arbitrarily.

A point regarding gravity-controlled gradient elution apparatus that seems to have escaped mention in the literature is that the liquid in the connecting tube must not mix by convexion with that in the mixer if the apparatus is to function accurately;

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[*] James A. Jobling & Co., England.

if such mixing does occur, one arm of the tube contains liquid that is denser than that in the other arm and the equilibrium heights of the liquids in the vessels are not simply determined by their densities. This is avoided by having the opening of the connecting tube into the mixer pointing either upwards or downwards depending on the relative densities of the two solvent components. However, the error incurred by the use of the wrong system is small if the connecting tube is not taken much above or below the level of the bottoms of the vessels. With the present apparatus (Fig. 2) these considerations do not apply, but it is necessary to prevent the contents of the vessels mixing by convexion, which is done by having the connecting tube lower than the reservoir at one point, as shown in Fig. 2.

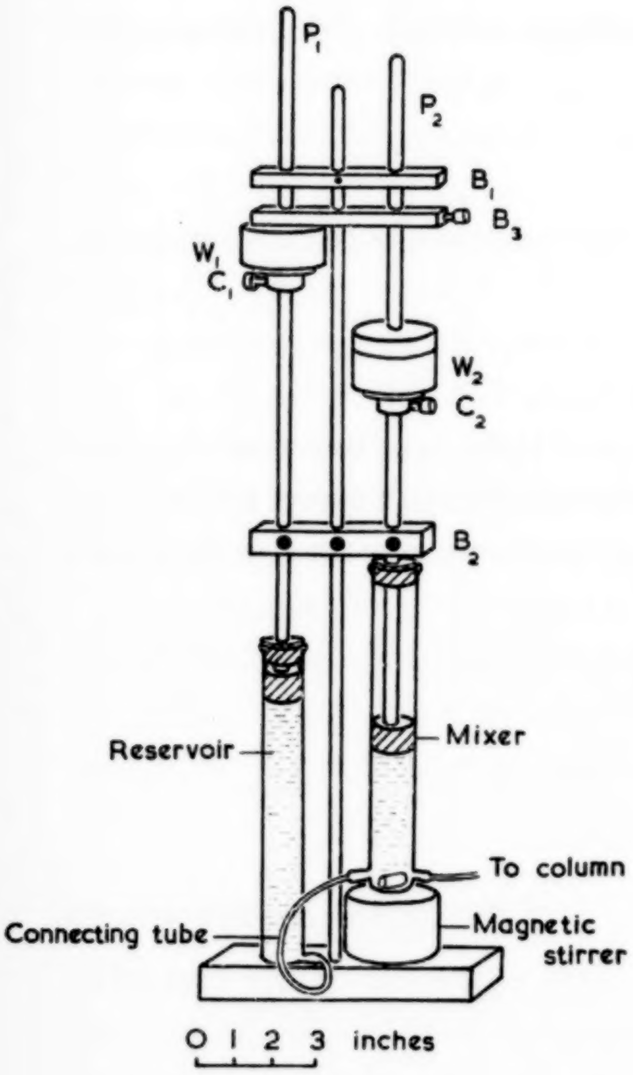

Fig. 2.

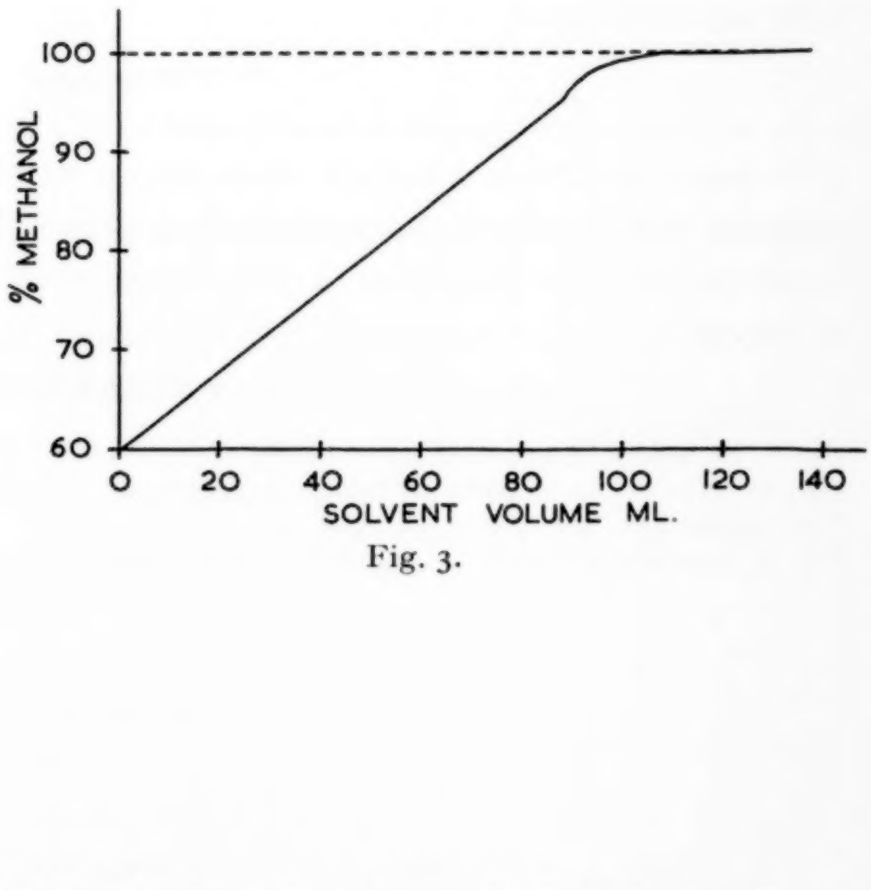

Fig. 3.

A disadvantage of the gradient elution device using pumps is that the gradient cannot be continued linearly quite up to the composition of the component in the reservoir because the stirrer index prevents the piston from going to the bottom of the mixer. In the present apparatus (Fig. 2) the reservoir continues to supply solvent after the pump in the mixer has stopped, so that the concentration of methanol in the effluent then proceeds asymptotically towards 100% (Fig. 3).

Another application of the PTFE-glass seal that has proved useful is as an adjustable solvent connector to a chromatographic column. The piston, which is fitted tightly on the end of a thick walled glass capillary tube, is pushed down the column onto the top of the packing; solvent is fed into the top of the glass capillary.

The advantage of this system is that the piston height can be adjusted to suit the height of the packing, practically eliminating the dead solvent space that occurs when ground joints are used.

Several other chromatographic applications suggest themselves. For example, the glass-teflon pump shown in Fig. 1 and the gradient elution apparatus (Fig. 2) could be easily adapted to supply solvent at a constant rate instead of a constant pressure by attaching a slow drive to the piston rods. Such a device would have the advantage over some reciprocating syringe-type metering pumps in that the supply of solvent would be continuous instead of intermittant. The pumping principle could also be applied to apparatus for producing complex gradients[3, 4]. Furthermore, the absolutely liquid-tight properties of the pump and the inertness of its materials would make it useful in a range of technical applications outside chromatography.

## ACKNOWLEDGEMENT

The assistance of Mr. E. FACER, who made some of the constant-bore tubes, is gratefully acknowledged.

## SUMMARY

This paper describes a simply made pump with a liquid-tight seal between the glass cylinder and the polytetrafluoroethylene piston. Its applications to column chromatography include the supplying of solvent under pressure, gradient elution and degassing of solvents.

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# SENSITIVITY OF THE FLUORESCENCE TEST FOR AMINO ACIDS*

J. OPIEŃSKA-BLAUTH, M. SANECKA AND M. CHAREZIŃSKI
*Biochemical Department, Medical Academy, Lublin (Poland)*

(Received August 5th, 1959)

The fluorescence test, when used for the detection of amino acids, is superior to other tests in that the chromatograms obtained can be used both for elution of unchanged amino acids from the filter paper, and for their identification by means of other colour reactions, whether general or specific. The general view is that amino acids fluoresce only on filter paper or on other cellulose materials, after being heated to 100–120°; according to most authors, the sensitivity of this test is much lower than that of the ninhydrin test.

The purpose of the present investigations was:

(a) to determine the sensitivity of the ordinary and sensitized u.v. tests for more than twenty amino acids;

(b) to study the influence of the temperature and heating time on the intensity of the fluorescence;

(c) to compare the sensitivity of the test before and after the development of the chromatogram in the systems phenol–water and propanol–water;

(d) to adapt the u.v. test, as has been done with the ninhydrin test, to the detection of amino acids in biological fluids.

## EXPERIMENTAL

### 1. *Materials and apparatus*

Standard amino acids of a high degree of purity.
Samples of physiological urines and sera.
Reagents sensitizing the u.v. test:

(a) 0.01 % xylose solution in ethanol used for soaking the filter paper.

(b) 0.01 % solution of sodium 1,2-naphthoquinone-4-sulphonate (NQS) in methanol used for soaking the filter paper.

U.v. lamp "Dedectolit" (filterglass OX1) emitting principally in the region 3500 Å.
U.v. lamp "Chromatolit" (filterglass OX7) emitting in the region 2537 Å.

### 2. *Procedure*

The term "specific sensitivity" of the test was applied to the smallest amount of a given amino acid (in $\mu$g) that produces a distinct fluorescence of the whole surface of

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* This work was aided by a grant from the Biological Division of the Polish Academy of Sciences.

the spot under strictly defined, standard experimental conditions. The standard conditions for the u.v. test are:

Filter paper: Whatman No. 1; volume of solution applied to the filter paper: 2.6 µl; area of the spot: 50.24 mm².

Developing solvents: phenol–water (7:3), propanol–water (7:3).

In order to determine the sensitivity after the development of the chromatogram, the area of the spot was measured with a planimeter and reduced to the starting surface.

The sensitivity of the test was determined either on untreated filter paper (ordinary test) or on filter paper soaked with a solution of NQS (sensitized test).

The comparison of the sensitivity of the u.v. test and of the ninhydrin reaction for the individual amino acids was based on the data contained in the paper by OPIEŃSKA-BLAUTH and co-workers[3].

TABLE I

SPECIFIC SENSITIVITIES OF AMINO ACIDS IN THE NINHYDRIN AND ISATIN REACTIONS AND IN THE ORDINARY AND SENSITIZED U.V. TESTS

| *No.* | *Amino acid* | $S_n$ µg | $S_{iz}$ µg | $S_{u.v.}$ µg | $S_{u.v._S}$ µg | $K_1$ | $K_2$ | $K_3$ |
|---|---|---|---|---|---|---|---|---|
| 1 | Alanine | 0.065 | 0.13 | 0.26 | 0.026 | 4.0 | 2.0 | 0.4 |
| 2 | β-Alanine | n.d. | 0.13 | 0.26 | 0.026 | — | 2.0 | — |
| 3 | α-Amino-*n*-butyric acid | n.d. | 1.04 | 0.26 | n.d. | — | 0.2 | — |
| 4 | Arginine | 0.13 | 0.78 | 0.26 | 0.026 | 2.0 | 0.3 | 0.2 |
| 5 | Asparagine | 0.26 | 1.04 | 0.32 | 0.26 | 1.2 | 0.3 | 1.0 |
| 6 | Aspartic acid | 0.065 | 0.78 | 0.32 | 0.026 | 5.0 | 0.4 | 0.4 |
| 7 | Glutamine | 0.13 | 0.26 | 0.32 | 0.052 | 2.4 | 1.2 | 0.4 |
| 8 | Glutamic acid | 0.03 | 0.13 | 0.32 | 0.026 | 10.6 | 2.4 | 0.86 |
| 9 | Glycine | 0.02 | 0.13 | 0.26 | 0.026 | 13.0 | 2.0 | 1.3 |
| 10 | Histidine | 0.13 | 0.26 | 0.32 | 0.026 | 2.4 | 1.2 | 0.2 |
| 11 | Hydroxyproline | n.d. | n.d. | 1.0 | 0.26 | — | — | — |
| 12 | Isoleucine | 0.065 | 0.26 | 0.32 | 0.13 | 5.0 | 1.2 | 0.2 |
| 13 | Leucine | 0.03 | 0.13 | 0.32 | 0.026 | 10.6 | 2.4 | 0.86 |
| 14 | Lysine | 0.065 | 0.26 | 0.13 | 0.13 | 2.0 | 0.5 | 0.2 |
| 15 | Methionine | 0.03 | 0.26 | 0.32 | 0.052 | 10.6 | 1.2 | 1.7 |
| 16 | Norleucine | 0.065 | 0.52 | 0.32 | 0.052 | 5.0 | 0.6 | 0.8 |
| 17 | Norvaline | 0.13 | 0.52 | 0.26 | 0.052 | 2.0 | 0.5 | 0.4 |
| 18 | Ornithine | 0.065 | 0.52 | 0.26 | 0.026 | 4.0 | 0.5 | 0.4 |
| 19 | Phenylalanine | 0.065 | 0.26 | 0.32 | 0.026 | 5.0 | 1.2 | 0.4 |
| 20 | Proline | 0.52 | 0.03 | 1.8 | 0.13 | 3.4 | 60.0 | 0.25 |
| 21 | Serine | 0.03 | 0.13 | 0.26 | 0.026 | 8.6 | 2.0 | 0.86 |
| 22 | Taurine | 0.52 | 1.04 | 0.26 | 0.052 | 0.5 | 0.2 | 0.1 |
| 23 | Threonine | 0.065 | 1.04 | 0.32 | 0.13 | 5.0 | 0.3 | 2.0 |
| 24 | Tryptophan | 0.065 | 0.26 | 0.032 | 0.026 | 0.5 | 0.12 | 0.4 |
| 25 | Valine | 0.03 | 0.13 | 0.32 | 0.026 | 10.6 | 2.4 | 0.86 |

$S$ = Sensitivity of the ninhydrin reaction.
$S_{iz}$ = Sensitivity of the isatin reaction.
$S_{u.v.}$ = Sensitivity of the fluorescence test.
$S_{u.v._S}$ = Sensitivity of the sensitized fluorescence test.

n.d. = not determined.

$$K_1 = \frac{S_{u.v.}}{S_n}; \qquad K_2 = \frac{S_{u.v.}}{S_{iz}}; \qquad K_3 = \frac{S_{u.v._S}}{S_n}.$$

Paper chromatography of the amino acids in serum was carried out after precipitation of proteins, while urine was desalted with an ion-exchange resin. Two-dimensional chromatograms were developed in the systems phenol–water and propanol–water. The spots were located by means of the u.v. lamp "Chromatolit".

## 3. *Results*

(A) A series of experiments, each of which was repeated several times, made it possible to determine the specific sensitivities of amino acids with regard to the u.v. test, both ordinary and sensitized (Table I). The coefficients $K_1$, $K_2$ and $K_3$ serve to compare the sensitivities of the individual amino acids in the ordinary and sensitized u.v. test with the sensitivities obtained in the ninhydrin and isatin reactions.

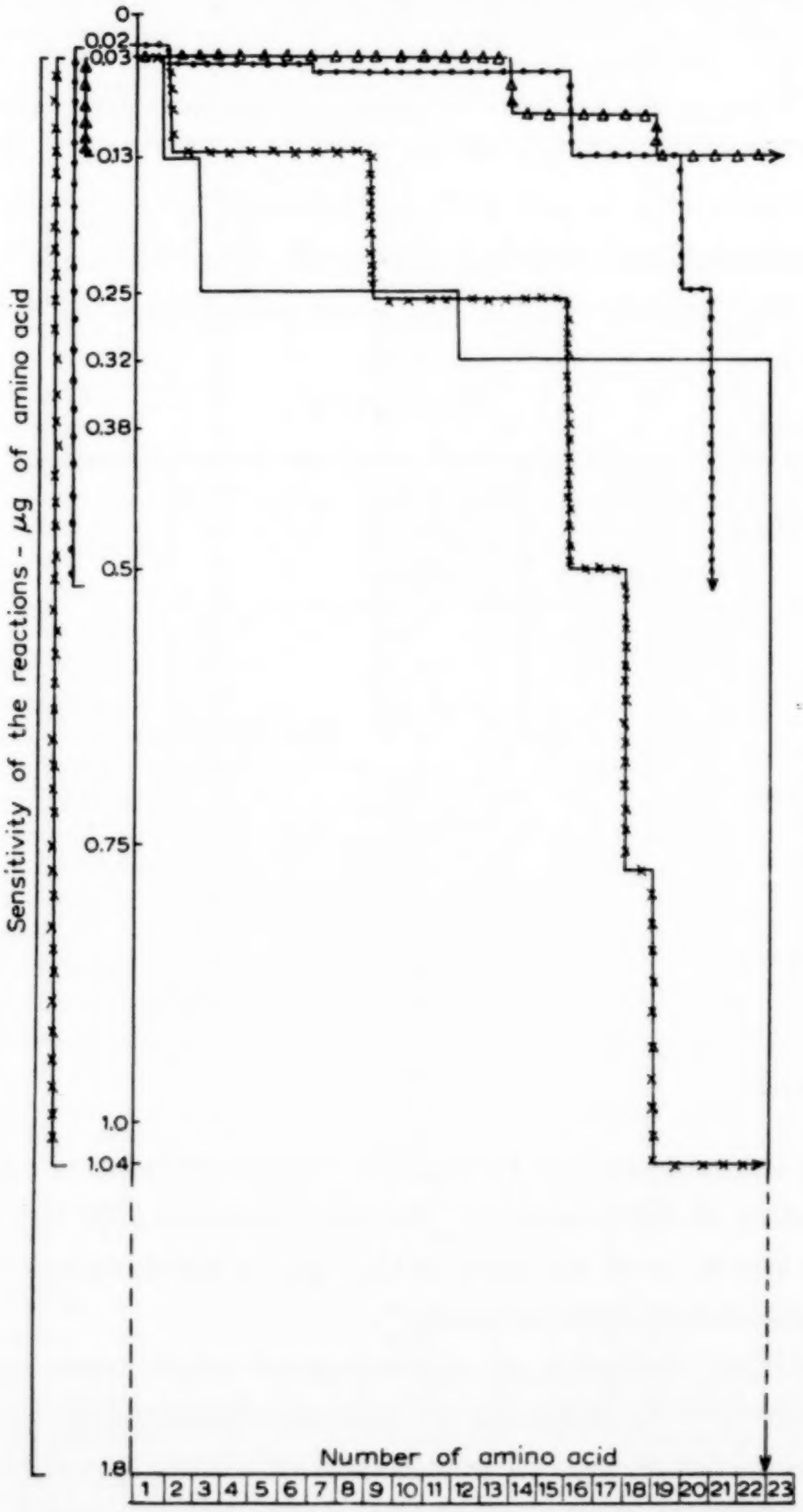

Fig. 1. Limits of sensitivity of amino acids. —●—●—●— ninhydrin reaction; —×—×—×— isatin reaction; ——— u.v. test; —△—△—△— sensitized u.v. test.

The sensitivity ranges of more than twenty amino acids in the ordinary and sensitized u.v. tests and in the ninhydrin and isatin reactions were also compared (Fig. 1).

(B) In order to determine the influence of various factors, such as temperature or time of heating of chromatograms before testing in u.v. light, comparative studies were carried out on amino acids not subjected to heating or heated to 70, 90, 110 and 120° for 2, 5 and 10 minutes (Tables II and III).

(C) The experiments resulted in the determination of the influence of the development of chromatograms in the systems phenol–water and propanol–water on the sensitivity of the u.v. tests (Tables IV and V). The coefficient $K$ expresses the relation of the sensitivity after development of the chromatogram to the specific sensitivity. In the calculations the ratio of the area of the spot after development to the standard area (50.24 mm²) was taken into account.

TABLE II

EFFECT OF TEMPERATURE OF HEATING ON THE SENSITIVITY OF THE FLUORESCENCE TEST

| | *Amino acid* | *μg* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *20°* | *40–50°* | *60–70°* | *80–90°* | *100–110°* | *110–120°* |
| 1 | Alanine | Lack of fluorescence | Fluorescence only on the periphery | 0.65 | 0.52 | 0.52 | 0.26 |
| 2 | β-Alanine | | | 0.52 | 0.37 | 0.37 | 0.26 |
| 3 | α-Amino-*n*-butyric acid | | | 0.65 | 0.52 | 0.32 | 0.26 |
| 4 | Arginine | | | 0.52 | 0.32 | 0.26 | — |
| 5 | Asparagine | | | n | 0.65 | 0.52 | 0.32 |
| 6 | Aspartic acid | | | n | n | n | 0.32 |
| 7 | Glutamine | | | n | 0.65 | 0.65 | 0.32 |
| 8 | Glutamic acid | | | n | 0.65 | 0.65 | 0.32 |
| 9 | Glycine | | | n | 0.65 | 0.65 | 0.26 |
| 10 | Histidine | | | n | n | 0.32 | 0.32 |
| 11 | Hydroxyproline | | | n | n | 2.6 | 1.0 |
| 12 | Isoleucine | | | n | n | n | 0.32 |
| 13 | Leucine | | | n | 0.65 | 0.52 | 0.32 |
| 14 | Lysine | | | 0.52 | 0.32 | 0.26 | 0.13 |
| 15 | Methionine | | | n | n | 0.65 | 0.32 |
| 16 | Norleucine | | | n | 0.65 | 0.65 | 0.32 |
| 17 | Norvaline | | | 0.52 | 0.32 | 0.32 | 0.26 |
| 18 | Ornithine | | | 0.32 | 0.26 | 0.26 | 0.26 |
| 19 | Phenylalanine | | | n | 0.65 | 0.52 | 0.32 |
| 20 | Proline | | | n | 5.2 | 2.6 | 1.8 |
| 21 | Serine | | | 0.52 | 0.32 | 0.32 | 0.26 |
| 22 | Taurine | | | n | 0.52 | 0.32 | 0.26 |
| 23 | Threonine | | | 0.52 | 0.32 | 0.32 | 0.32 |
| 24 | Tryptophan | | | n | n | 0.52 | 0.032 |
| 25 | Valine | | | 0.65 | 0.52 | 0.52 | 0.32 |

n = lack of fluorescence.

(D) After desalting, samples of physiological urine were applied to the filter paper in various quantities, from 25 to 100 μl. Protein-free samples of sera were applied to the filter paper in 250 μl portions. Two-dimensional chromatograms were developed in the systems propanol–water and phenol–water. Amino acids were visualized by means of the u.v. tests and the ninhydrin reaction (Table VI).

## DISCUSSION AND CONCLUSIONS

The importance of the fluorescence test for the detection of amino acids and its superiority to the ninhydrin reaction have been stressed by Phillips[5] and Woiwod[10,11] Phillips gives the value of about 20 µg as the lower limit of the sensitivity of the u.v. test for most amino acids studied by him. Broadly speaking, most authors stress the low sensitivity of the u.v. reaction. According to Woiwod, tryptophan, histidine, and citrulline are exceptions, their fluorescence sensitivity being much greater than that of other amino acids.

The phenomenon of the amino acid fluorescence in u.v. light is undoubtedly connected with cellulose. According to Woiwod, the mechanism of the fluorescence is based on the reaction of the amino group of amino acids with the aldehyde group of saccharides[8]. The fluorescence appears only after heating. This is confirmed by Woiwod's experiments with cellulose powders[10] and by Graham's[1] experiments with glucose and 15 amino acids. Shore and Pardee[6] increased the intensity of the fluorescence of amino acids spots by treating the filter paper with a xylose solution before placing on it the amino acid samples. Kofranyi[2] obtained excellent results in increasing the intensity of the amino acid fluorescence in u.v. light by means of another sensitizing

TABLE III

EFFECT OF HEATING TIME ON THE SENSITIVITY OF THE FLUORESCENCE TEST

| | *Amino acid* | *µg* | | |
|---|---|---|---|---|
| | | *2 min* | *5 min* | *10 min* |
| 1 | Alanine | n | 0.37 | 0.26 |
| 2 | β-Alanine | 0.52 | 0.26 | 0.26 |
| 3 | α-Amino-*n*-butyric acid | n | 0.52 | 0.26 |
| 4 | Arginine | n | 0.32 | 0.26 |
| 5 | Asparagine | n | n | 0.32 |
| 6 | Aspartic acid | n | n | 0.32 |
| 7 | Glutamine | n | 0.52 | 0.32 |
| 8 | Glutamic acid | 0.65 | 0.65 | 0.32 |
| 9 | Glycine | n | 0.32 | 0.26 |
| 10 | Histidine | n | n | 0.32 |
| 11 | Hydroxyproline | n | 1.7 | 1.0 |
| 12 | Isoleucine | n | 0.65 | 0.32 |
| 13 | Leucine | 0.65 | 0.65 | 0.32 |
| 14 | Lysine | 0.65 | 0.26 | 0.13 |
| 15 | Methionine | n | n | 0.32 |
| 16 | Norleucine | 0.65 | 0.65 | 0.32 |
| 17 | Norvaline | 0.52 | 0.32 | 0.26 |
| 18 | Ornithine | 0.52 | 0.32 | 0.26 |
| 19 | Phenylalanine | n | 0.32 | 0.32 |
| 20 | Proline | 3.4 | 2.6 | 1.8 |
| 21 | Serine | 0.52 | 0.32 | 0.26 |
| 22 | Taurine | n | 0.32 | 0.26 |
| 23 | Threonine | n | 0.52 | 0.32 |
| 24 | Tryptophan | 0.26 | 0.13 | 0.032 |
| 25 | Valine | 0.52 | 0.32 | 0.32 |

n = lack of fluorescence.

TABLE IV

SENSITIVITY OF THE U.V. TEST APPLIED TO CHROMATOGRAMS DEVELOPED IN THE SYSTEM PHENOL–WATER

| | *Amino acid* | $S_A$ μg | $S_{ph}$ μg | $P_{ph}$ mm² | $S_R'$ μg | $K_{ph}$ |
|---|---|---|---|---|---|---|
| 1 | Alanine | 0.26 | 0.65 | 70 | 0.46 | 1.7 |
| 2 | β-Alanine | 0.26 | 0.43 | 140 | 0.16 | 0.6 |
| 3 | α-Amino-*n*-butyric acid | 0.26 | 0.85 | 110 | 0.38 | 1.4 |
| 4 | Arginine | 0.26 | 1.04 | 85 | 0.61 | 2.3 |
| 5 | Asparagine | 0.32 | 0.85 | 100 | 0.42 | 1.3 |
| 6 | Aspartic acid | 0.32 | 0.85 | 70 | 0.60 | 1.8 |
| 7 | Glutamine | 0.32 | 1.3 | 130 | 0.50 | 1.5 |
| 8 | Glutamic acid | 0.32 | 0.65 | 70 | 0.46 | 1.4 |
| 9 | Glycine | 0.26 | 0.65 | 100 | 0.32 | 1.2 |
| 10 | Histidine | 0.32 | 0.85 | 90 | 0.47 | 1.4 |
| 11 | Hydroxyproline | 1.05 | 12.48 | 270 | 2.8 | 2.4 |
| 12 | Isoleucine | 0.32 | 1.7 | 110 | 0.77 | 2.4 |
| 13 | Leucine | 0.32 | 1.48 | 110 | 0.67 | 2.0 |
| 14 | Lysine | 0.13 | 0.52 | 130 | 0.20 | 1.5 |
| 15 | Methionine | 0.32 | 1.3 | 110 | 0.59 | 1.8 |
| 16 | Norleucine | 0.32 | 1.48 | 80 | 0.92 | 2.8 |
| 17 | Norvaline | 0.26 | 1.48 | 140 | 0.53 | 2.0 |
| 18 | Ornithine | 0.26 | 0.65 | 60 | 0.54 | 2.0 |
| 19 | Phenylalanine | 0.32 | 1.7 | 100 | 0.85 | 2.6 |
| 20 | Proline | 1.8 | 15.5 | 250 | 3.1 | 1.7 |
| 21 | Serine | 0.26 | 0.52 | 120 | 0.21 | 0.8 |
| 22 | Taurine | 0.26 | 0.32 | 130 | 0.12 | 0.4 |
| 23 | Threonine | 0.32 | 0.85 | 100 | 0.42 | 1.3 |
| 24 | Tryptophan | 0.032 | 1.3 | 120 | 0.55 | 16.0 |
| 25 | Valine | 0.32 | 1.3 | 100 | 0.65 | 2.0 |

$S_A$ = Specific sensitivity.
$S_{ph}$ = Sensitivity after development.
$P_{ph}$ = Area of the spot.
$S_R'$ = Reduced sensitivity after development.

$$K_{ph} = \frac{S_R'}{S_A} ; \qquad S_R' = S_{ph} \frac{50.24}{P_{ph}} .$$

reagent belonging to the group of naphthoquinone compounds. He does not explain, however, the mechanism by which 1,2-naphthoquinone-4-sulphonate renders the filter paper more sensitive. In any case, a stronger contrast is produced between the amino acid spots and the remaining surface of the filter paper. VEN HORST and co-workers[9] carried out quantitative determinations of the intensity of the amino acid fluorescence in u.v. light using a photodensitometer specially adapted to this purpose.

We were interested in several questions connected with the u.v. test. One of them was the problem of differences in its sensitivity for the individual amino acids, since investigations on the amino acid composition of substances are considerably hindered by the fact that the sensitivity ranges for the different amino acids in the ninhydrin, isatin and alloxane reactions oscillate within broad limits.

The second question was the comparison between the sensitivity of the u.v. test for amino acids and the sensitivity of other tests, especially of the generally used ninhydrin reaction. It is probable that a combination of both methods will allow

TABLE V

SENSITIVITY OF THE U.V. TEST APPLIED TO CHROMATOGRAMS DEVELOPED IN THE SYSTEM PROPANOL–WATER

| | *Amino acid* | $S_A$ μg | $S_{pr}$ μg | $P_{pr}$ mm² | $S_R'$ μg | $K_{pr}$ |
|---|---|---|---|---|---|---|
| 1 | Alanine | 0.26 | 0.37 | 110 | 0.17 | 0.65 |
| 2 | β-Alanine | 0.26 | 0.37 | 100 | 0.18 | 0.69 |
| 3 | α-Amino-*n*-butyric acid | 0.26 | 0.85 | 90 | 0.46 | 1.76 |
| 4 | Arginine | 0.26 | 0.65 | 90 | 0.40 | 1.53 |
| 5 | Asparagine | 0.32 | 0.65 | 100 | 0.32 | 1.0 |
| 6 | Aspartic acid | 0.32 | 0.85 | 90 | 0.46 | 1.42 |
| 7 | Glutamine | 0.32 | 1.04 | 100 | 0.52 | 1.62 |
| 8 | Glutamic acid | 0.32 | 0.65 | 90 | 0.40 | 1.25 |
| 9 | Glycine | 0.26 | 0.26 | 90 | 0.14 | 0.53 |
| 10 | Histidine | 0.32 | 0.65 | 70 | 0.52 | 1.62 |
| 11 | Hydroxyproline | 1.05 | 12.48 | 240 | 2.3 | 2.1 |
| 12 | Isoleucine | 0.32 | 1.48 | 70 | 1.06 | 3.31 |
| 13 | Leucine | 0.32 | 1.7 | 70 | 1.21 | 3.36 |
| 14 | Lysine | 0.13 | 0.13 | 75 | 0.08 | 0.61 |
| 15 | Methionine | 0.32 | 1.3 | 70 | 0.93 | 2.90 |
| 16 | Norleucine | 0.32 | 1.48 | 80 | 0.92 | 2.87 |
| 17 | Norvaline | 0.26 | 1.3 | 90 | 0.72 | 2.69 |
| 18 | Ornithine | 0.26 | 0.52 | 130 | 0.20 | 0.76 |
| 19 | Phenylalanine | 0.32 | 1.3 | 75 | 0.87 | 2.71 |
| 20 | Proline | 1.8 | 13.0 | 250 | 2.8 | 1.4 |
| 21 | Serine | 0.26 | 0.85 | 110 | 0.38 | 1.18 |
| 22 | Taurine | 0.26 | 0.52 | 70 | 0.37 | 1.15 |
| 23 | Threonine | 0.32 | 1.3 | 110 | 0.67 | 2.09 |
| 24 | Tryptophan | 0.032 | 0.65 | 125 | 0.26 | 8.12 |
| 25 | Valine | 0.32 | 1.3 | 70 | 0.93 | 2.90 |

$S_A$ = Specific sensitivity.
$S_{pr}$ = Sensitivity after development.
$P_{pr}$ = Area of the spot.
$S_R'$ = Reduced sensitivity after development.

$$K_{pr} = \frac{S_R'}{S_A}\,; \qquad S_R' = S_{pr}\,\frac{50.24}{P_{pr}}\,.$$

detection and identification in chromatograms of quite small amounts of those amino acids whose sensitivities in both tests are considerably different.

The third and last question concerned the prospects of finding the optimal experimental conditions for the u.v. test which might make possible the detection and identification of amino acids occurring in various concentrations in biological fluids.

The present investigations were based on the method used for determination of sensitivity in the ninhydrin, isatin and alloxane reactions[3, 4, 7].The smallest amount of an amino acid which under standard experimental conditions produces fluorescence of the whole standard area of the spot was considered as the specific sensitivity. As Table I and Fig. 1 show, the sensitized u.v. test best meets our requirements. The specific sensitivities range here between 0.03 and 0.13 μg, the ordinary u.v. test shows a broader range: 0.03 to 1.8 μg. In the ninhydrin test the specific sensitivities range between 0.02 and 0.52 μg, and in the isatin reaction between 0.03 and 1.0 μg. Detailed

## TABLE VI

DETECTION OF AMINO ACIDS IN URINE AND SERUM SAMPLES BY MEANS OF THE U.V. TEST AND THE NINHYDRIN TEST

| | *Amino acid* | *25 µl urine* | | | | | *50 µl urine* | | | | | *100 µl urine* | | | | | *250 µl serum* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *u.v.* | $N_s$ | $u.v._s$ | $N_{ss}$ | *N* | *u.v.* | $N_s$ | $u.v._s$ | $N_{ss}$ | *N* | *u.v.* | $N_s$ | $u.v._s$ | $N_{ss}$ | *N* | *u.v.* | $N_s$ | $u.v._s$ | $N_{ss}$ | *N* |
| 1 | Glycine | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 2 | Serine | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 3 | Histidine | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 4 | Alanine | — | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 5 | Threonine | — | + | — | — | + | — | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 6 | Lysine | — | + | + | — | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 7 | Glutamine | — | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 8 | Tyrosine | — | — | + | — | + | — | + | + | — | + | + | + | + | + | + | + | + | + | + | + |
| 9 | Valine | — | — | + | — | — | — | + | + | — | + | — | + | + | — | + | + | + | + | + | + |
| 10 | Asparagine | — | — | — | — | — | + | — | + | — | + | + | + | + | + | + | + | + | + | + | + |
| 11 | Aspartic acid | — | — | — | — | — | — | — | — | — | + | — | — | — | — | + | + | + | + | + | + |
| 12 | Glutamic acid | — | + | + | — | + | — | + | + | + | + | — | + | + | + | + | + | + | + | + | + |
| 13 | Lysine | — | — | — | — | + | — | — | + | — | + | — | — | + | + | + | + | + | + | — | — |
| 14 | Leucine | — | — | — | — | — | — | — | — | — | — | — | + | + | — | + | + | + | + | + | + |
| 15 | *α*-Amino-*n*-butyric acid | — | — | — | — | — | — | — | — | — | — | + | — | + | + | + | — | — | + | — | — |
| 16 | *β*-Amino-isobutyric acid | — | — | — | — | — | — | — | — | — | — | — | + | + | — | + | — | — | — | — | — |
| 17 | Yellow spot | — | — | — | — | — | — | + | + | — | + | — | + | + | + | + | — | — | — | — | — |
| 18 | Spot a | — | — | — | — | — | — | — | — | — | — | — | + | — | + | + | + | + | — | — | — |
| 19 | Spot b | — | — | — | — | + | — | — | — | — | + | + | + | + | + | + | + | + | + | + | + |
| 20 | Total | 3 | 8 | 9 | 5 | 11 | 7 | 11 | 13 | 8 | 15 | 11 | 16 | 17 | 15 | 19 | 16 | 16 | 16 | 14 | 14 |

$N_s$ = Ninhydrin test subsequent to u.v. test.
$N_{ss}$ = Ninhydrin test subsequent to $u.v._s$ test.
N = Ninhydrin test alone.

studies show that there are comparatively great differences in the sensitivity of the u.v. test and of the ninhydrin and isatin reactions for the individual amino acids. For example, in the ninhydrin reaction glycine shows the greatest specific sensitivity (0.02 $\mu$g), proline and taurine the lowest (0.52 $\mu$g). In the u.v. test the highest specific sensitivity is shown by tryptophan (0.03 $\mu$g) and the lowest by proline (1.8 $\mu$g). The absence of the proper amino group in proline and hydroxyproline explains the weak fluorescence of these amino acids. In most of the amino acids we studied, the specific sensitivity of the sensitized u.v. test was nearly ten times higher than that of the ordinary u.v. test. Only a few amino acids, such as asparagine, lysine and tryptophan, were a definite exception to this (Table I).

The results of the present investigations on the optimal conditions for the u.v. test with regard to temperature and time of heating before testing the fluorescence correspond to the results obtained by VEN HORST and co-workers. The optimal temperature for heating chromatograms was found to be 110–120°, the optimum heating time 10 minutes. When the heating temperatures are lower than 50°, none of the amino acids studied produces fluorescence; only when heated to 110–120° for 10 minutes, do all amino acids fluoresce (Tables II and III).

Our previous comparative investigations on the sensitivity of the ninhydrin reaction before and after development of the chromatograms gave different results for the individual amino acids. These differences are caused by losses of amino acids during their migration in the filter paper.

The greatest losses were observed in amino acids with the highest $R_F$ values. Factors probably contributing to these losses are: adsorption on the filter paper, decomposition of the substances during migration, chemical interaction of the amino acids and solvents, and impurities contained in the filter paper. The u.v. test also showed losses of amino acids during the development of chromatograms.

Comparison of the results expressed in the coefficients $K_{\mathrm{phen.}}$ disclosed that the losses are the greatest in tryptophan ($K_{\mathrm{ph.}} = 16, K_{\mathrm{pr.}} = 8$), and the smallest in taurine ($K_{\mathrm{ph.}} = 0.4$) and lysine ($K_{\mathrm{pr.}} = 0.6$).

The identification of amino acids contained in urine and blood serum can be carried out by means of the sensitized u.v. test and the ninhydrin test applied successively.

When the u.v. and ninhydrin tests were applied successively in order to detect amino acids in chromatograms of urine and blood serum (Table VI), the results obtained in both tests were more or less identical, providing that the volumes of urine placed on the filter paper were not too small (not below 100 $\mu$l).

The ordinary u.v. test is undoubtedly less sensitive for the majority of amino acids than the ninhydrin reaction; that is why in small volumes of urine (25 $\mu$l) the u.v. test is unable to detect all those amino acids that can be detected by means of the ninhydrin reaction.

The sensitized u.v. test, on the other hand, equals the ninhydrin reaction as far as sensitivity is concerned, but it involves greater losses of amino acids. The losses are especially marked when small volumes of urine (25 $\mu$l) are placed on the filter paper. Successive application of both tests, one immediately after the other, though very convenient, may therefore be made use of only for qualitative and approximately quantitative estimation of amino acids.

The numerical data presented in the tables are by no means constant characteristic values; neither can they be exactly reproduced in further experiments. Even when all other experimental conditions were the same, the use of two lamps of the same type gave different results. The age of the filter is probably also of importance.

Thus the values tabulated in this article should be regarded as examples to be used for comparative purposes.

## SUMMARY

Comparative investigations were carried out on the sensitivity of the ordinary and sensitized u.v. test for amino acids. To sensitize the test the filter paper was impregnated with 1,2-naphthoquinone-4-sulphonate. In the experiments, the u.v. lamp with Wood's filter (3500 Å) and the "Chromatolit" lamp (2537 Å) were used. For most of the amino acids investigated the sensitivity of the ordinary u.v. test was lower than that of the ninhydrin reaction. The sensitivity of the sensitized u.v. test proved to be ten times higher than that of the ordinary u.v. test for the majority of amino acids. Only in the case of asparagine, lysine and tryptophan was the increase of sensitivity slight. As with the ninhydrin and isatin reactions, the development of the chromatograms has an unfavourable influence on the sensitivity of the u.v. test. Greatest losses were observed in amino acids with high $R_F$ coefficients, such as tryptophan, leucine, methionine and valine.

The optimal experimental conditions for the u.v. test were determined; chromatograms should be heated to 110–120°, and the time of heating should be 10 minutes.

The identification of amino acids contained in urine and blood can be carried out by means of the common or sensitized u.v. tests and the ninhydrin test applied successively, providing that the volumes of urine applied are not too small (about 100 $\mu$l).

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# ELECTROPHORETIC SEPARATION OF SOME AMINO ACIDS FROM THEIR COPPER COMPLEXES*

M. SZWAJ AND M. KAŃSKI
*Biochemical Department, Medical Academy, Lublin (Poland)*

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Chelate compounds of amino acids with copper ion have been studied for many years[1-4].

It has been stated that copper ion combines with monocarboxylic α-amino acids, giving compounds of the type $Cu(A)_2$, where A = amino acid, and with glutamic acid, aspartic acid, isoserine and polypeptides giving compounds of the type CuA[5,6].

The equilibrium constants of the reactions between heavy metal ions and the amino acids containing two ionizable groups[7] and those containing three ionizable groups[8] were also determined. MILLS[9] used paper chromatography for the separation of copper ions from organic complexes occurring in the aqueous extracts of green Australian herbage.

CRUMPLER AND DENT[10], using the same technique, found that β- and γ-amino acids were unable to react with copper.

BECK AND CSÁSZAR[11] have succeeded in separating some of the copper–amino acid complexes by paper chromatography; most of them however, had very close $R_F$ values.

Separation of amino acids into three major groups (acidic, neutral and basic) employing low-voltage (gradient potential of 5 V/cm) paper electrophoresis was achieved as early as 1948 by WIELAND *et al.*[12].

DOBBIE *et al.*[13-15] have studied copper complexes with glycine, glycylglycine, glycyl-L-leucine, glycyl-L-tyrosine, carnosine and diglycylglycine using potentiometric titration, copper-electrode potential measurement, electrophoretic mobilities on Whatman No. 54 paper and spectrophotometric observations.

Our preliminary study on the resolution of some amino acids and their copper complexes by paper chromatography gave rather unsatisfactory results. The application of paper electrophoresis, however, proved to be more promising.

In this paper the results of the separation of a number of free amino acids from their copper complexes by the paper electrophoresis method are presented.

## EXPERIMENTAL

### *Materials*

The following reagents were used throughout this work: sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$) c.p., cupric chloride ($CuCl_2 \cdot 2H_2O$) c.p., amino acids (L. Light & Co., Ltd.),

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ninhydrin (British Drug Houses Ltd.), rubeanic acid (Merck). The paper strips (Whatman No. 4) were 30 cm long and 4 cm wide.

### *Apparatus*

The apparatus used was a conventional horizontal one made of plexiglass with tight cover-plate, operating in the potential range 0–400 V d.c. and at a current strength in the range 0–100 mA.

### *Methods*

Amino acid–cupric complexes were prepared according to NEUBERG, LUSTIG AND MANDL[16] with the following amino acids: glycine, serine, threonine, asparagine, glutamine, aspartic acid, glutamic acid, lysine and ornithine. Amino nitrogen was determined according to POPE AND STEVENS[17]. Determinations of copper were performed colorimetrically with the use of diethyl dithiocarbamate[18]. The $\mathrm{Cu/N_{NH_2}}$ ratio indicated that the complexes obtained in this way have the formula mentioned above[5,6]. The following conditions were taken into account in the preliminary experiments: potential gradient, ionic strength, duration of electrophoretic process, and quality of the buffer employed. Three buffers were chosen:

(1) 0.025 $M$ sodium tetraborate + 0.05 $M$ NaOH, pH = 10, $\mu = 0.05$.
(2) 0.025 $M$ sodium tetraborate + 0.05 $M$ HCl, pH = 9.1, $\mu = 0.05$.
(3) 0.025 $M$ sodium tetraborate + 0.1 $M$ $H_3BO_3$, pH = 9.1, $\mu = 0.05$.

The most satisfactory results were obtained at the potential gradient 10 V/cm of paper strip length, 0.7 mA/cm of strip width and 1.5 h electrophoresis. Whatman

TABLE I

ELECTROPHORETIC MIGRATION RATES ($R$) UNCORRECTED AND CORRECTED FOR ELECTROENDOSMOTIC EFFECT

| *Amino acid or copper complex* | *R in mm (uncorrected)* | *R in mm (corrected for electroendosmotic effect)* |
|---|---|---|
| Glycine | + 15.3 | + 38.3 |
| Cu–glycine | — 11.4 | + 11.6 |
| Serine | + 24.2 | + 47.2 |
| Cu–serine | — 7.5 | + 15.5 |
| Threonine | + 25.5 | + 48.5 |
| Cu–threonine | — 10.0 | + 13.0 |
| Asparagine | + 29.1 | + 52.1 |
| Cu–asparagine | — 8.7 | + 14.3 |
| Glutamine | + 20.5 | + 43.5 |
| Cu–glutamine | — 10.0 | + 13.0 |
| Aspartic acid | + 55.4 | + 78.4 |
| Cu–aspartic acid | + 63.9 | + 86.9 |
| Glutamic acid | + 50.7 | + 73.7 |
| Cu–glutamic acid | + 57.7 | + 80.0 |
| Lysine | — 36.9 | — 13.9 |
| Cu–lysine | — 50.7 | — 27.7 |
| Ornithine | — 25.5 | — 2.5 |
| Cu–ornithine | — 37.7 | — 14.7 |
| Creatinine | — 23.0 | |

No. 4 strips 30 × 4 cm were placed on the supporting part of the electrophoresis chamber and the solutions to be separated (10–15 μg) were applied to the centre line across the paper strip. After the electrophoretic process was finished the paper strips were taken out, dried horizontally in an oven at 105° on a glass supporting frame for 10 minutes. The dried paper strips were cut along the long axis; one part was sprayed with 10–15 % solution of acetic acid in acetone, dried at room temperature for approx. 15 minutes and developed with 0.1 % ninhydrin (300 ml of methyl alcohol + 185 ml of *n*-butanol + 15 ml of 2 *N* acetic acid) at 100–105°; the other part was sprayed with 0.1 % solution of rubeanic acid in methanol (the positive reaction for copper appears almost immediately).

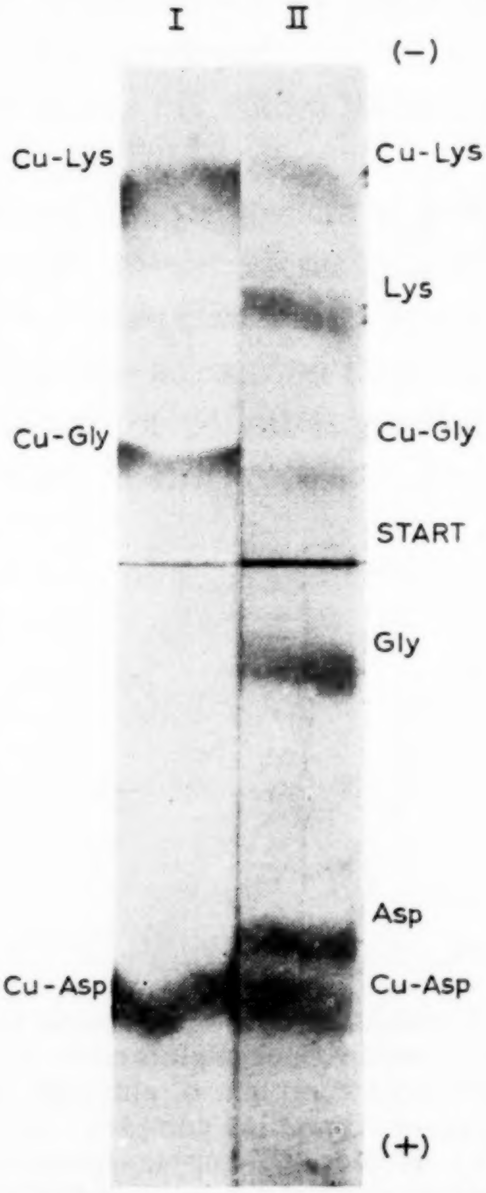

Fig. 1. Separation of glycine, lysine and aspartic acid from their copper complexes. Experimental conditions: borate–NaOH buffer, pH 10, μ 0.05, duration of electrophoresis 1.5 h, potential gradient 10 V/cm. I, paper strip developed with rubeanic acid; II, paper strip developed with ninhydrin.

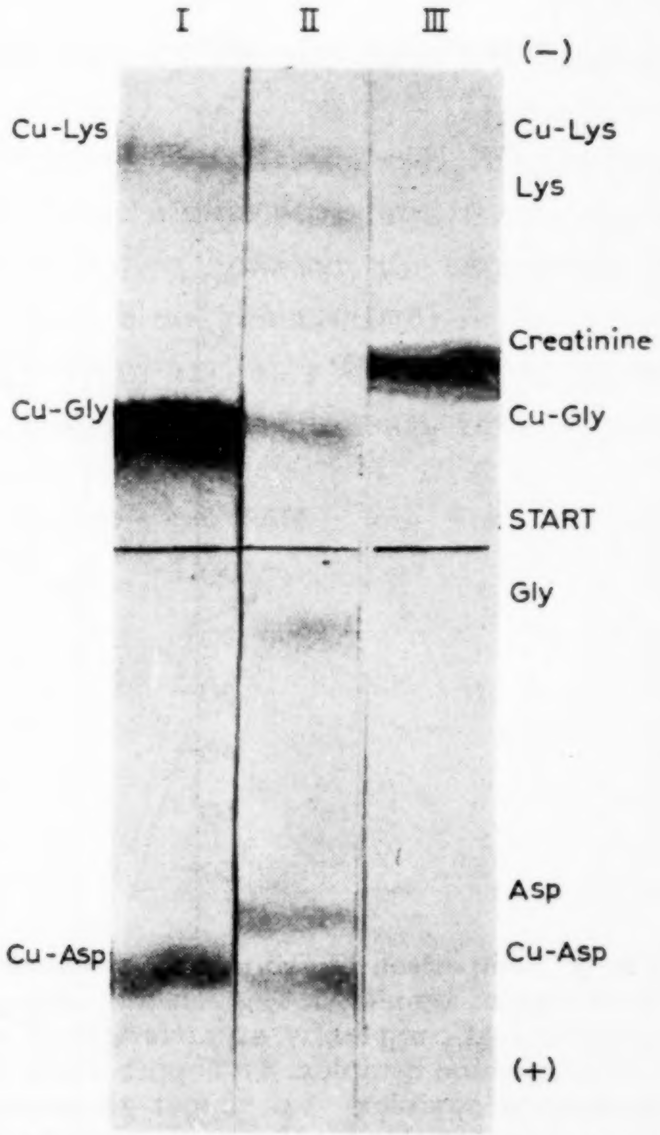

Fig. 2. As in Fig. 1 with creatinine added (serving as a control for electroendosmotic effect). Experimental conditions as in Fig. 1. I and II as in Fig. 1; III, paper strip developed with alkaline picrate for creatinine.

The experiments concerning the applicability of the alkaline borate buffers were performed using three amino acids (glycine, lysine, aspartic acid) and their copper complexes. The most useful buffer appeared to be the borate–NaOH buffer (pH 10, μ 0.05), as indicated by the calculated electrophoretic migration rates of the substances tested. The electrophoretic mobilities were calculated in millimetres and recorded as

(+) or (—) with respect to localization of a detected compound at the anode or cathode zone respectively of the paper strip (Table I).

The results of the separation of some of the compounds studied are shown in Figs. 1, 2 and 3.

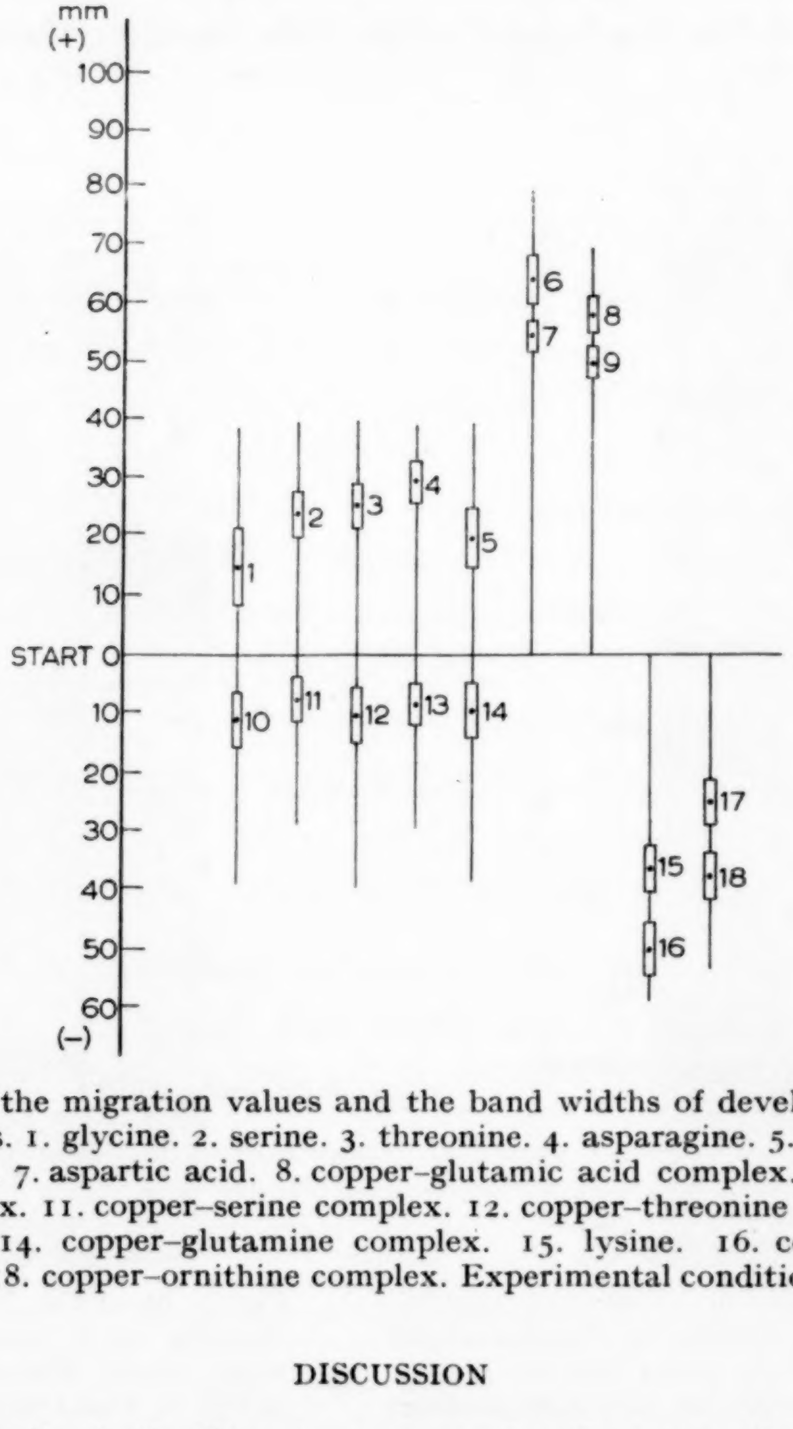

Fig. 3. Comparison of the migration values and the band widths of developed amino acids and their copper complexes. 1. glycine. 2. serine. 3. threonine. 4. asparagine. 5. glutamine. 6. copper–aspartic acid complex. 7. aspartic acid. 8. copper–glutamic acid complex. 9. glutamic acid. 10. copper–glycine complex. 11. copper–serine complex. 12. copper–threonine complex. 13. copper–asparagine complex. 14. copper–glutamine complex. 15. lysine. 16. copper–lysine complex. 17. ornithine. 18. copper–ornithine complex. Experimental conditions as in Fig. 1.

## DISCUSSION

There is little information in the literature concerning the separation of free amino acids from their copper complexes. The resolution of amino acids and of amino acid–copper complexes on separate paper electropherograms was reported by WIELAND *et al.*[12].

DOBBIE *et al.*[13-15] reported electrophoretic mobilities of copper complexes with glycine and some dipeptides, without attempting to separate a larger number of amino acid–copper complexes.

In this report the results of the application of low-voltage paper electrophoresis for the purpose of separating some free amino acids from their copper complexes are described.

BORSOOK[19] has demonstrated that the complexes of the type Cu(A)$_n$ can be formed not only in the alkaline but also in the acid range of pH. We are able to state that at pH 5–6 in acetate buffer all the complexes tested are liable to considerable decomposition, while in phosphate buffer the stability of basic amino acid complexes is lost almost completely.

Below pH 7, there was no resolution between free amino acids and their copper complexes since both had the same migration rate (*R*) values. The optimal separation is reached in the pH range of 9–11.

In WIELAND'S work cited above[12] only 0.1 *M* acetate buffer at pH range of 3.7–7.5 was employed.

At higher pH values the detection of amino acids by the ninhydrin test becomes increasingly difficult.

Of the borate buffers at pH 9–11 the borate–HCl and borate–$H_3BO_3$ buffers gave better results as far as the resolution of basic amino acids and their copper complexes were concerned; in the borate–NaOH buffer, however, the separation of neutral amino acids from their complexes was more clearly demonstrable.

Changes of potential gradient between 3.3 V/cm and 10.2 V/cm are of minor importance in the process of zone separation, providing the necessary time adjust-

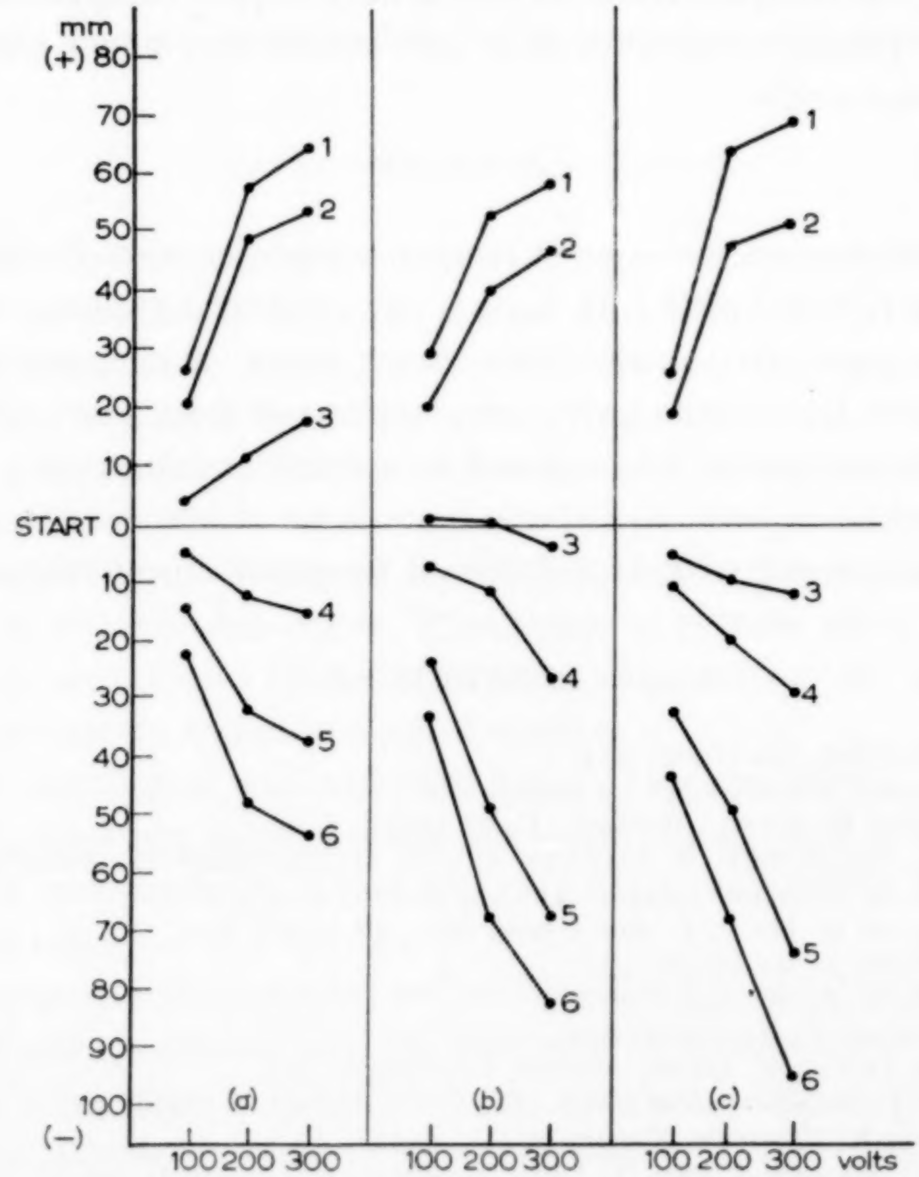

Fig. 4. Dependence of migration values of amino acids and their copper complexes on the potential applied. 1. copper–aspartic acid complex. 2. aspartic acid. 3. glycine. 4. copper–glycine complex. 5. lysine. 6. copper–lysine complex. Experimental conditions: (a) borate–NaOH buffer, pH 10; (b) borate–HCl buffer, pH 9.1; (c) borate–$H_3BO_3$ buffer, pH 9.1. $\mu$ 0.05, duration of electrophoresis 1.5 h, Whatman No. 4 paper strips 30 × 4 cm. Ordinates: migration rates in millimetres. Abscissae: potential applied in volts (100, 200 and 300 V correspond to gradient potentials: 3.3 V/cm, 6.6 V/cm and 10 V/cm).

ments are made. Fig. 4 would suggest that the potential gradients ranging between 6.6 and 10.2 V/cm are the most appropriate.

When one places a solution of amino acid on the starting line of a paper strip, followed by a solution of copper salt, apparently the same complexes are formed as those prepared by the classical methods.

On applying to the paper a mixture of an amino acid–copper complex with another amino acid, a partial displacement of the complexed amino acid occurs and a new complex is formed. The competitive interaction is observed throughout the whole range of pH studied, *viz.* 5–11.

Similar observations have been made by WIELAND *et al.*[12] in the case of (histidine)$_2$–Cu complex capable of exchanging one histidine molecule with another amino acid molecule and forming in this way a mixed amino acid–Cu complex.

Under our conditions it was possible to separate amino acid–copper complexes into three main groups: acidic, neutral and alkaline, and in each group to separate a given complex from its amino acid.

If necessary, the composition of an amino acid–copper complex can be determined following copper precipitation with $H_2S$ and subsequent paper chromatography of the liberated amino acid.

## SUMMARY

Mixtures of free amino acids and their copper complexes were fractionated by paper electrophoresis in borate buffer (pH 10, $\mu$ 0.05), potential gradient 6.6–10.2 V/cm on Whatman No. 4 paper strips into three major zones: acidic, neutral and alkaline. Within these zones free amino acids were separated from their copper complexes. Below pH 5–6 the complexes decomposed to a considerable extent.

The amino acid composition of the common zone of complexes may be determined by the removal of copper with $H_2S$ followed by paper chromatography of liberated amino acids.

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# CHROMATOGRAPHIC DETERMINATION OF CYSTEIC ACID*

SELMA L. BANDEMER AND ROBERT JOHN EVANS
*Department of Agricultural Chemistry, Michigan State University, East Lansing, Mich. (U.S.A.)*

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The instability of cystine in protein materials during acid hydrolysis has led to the development of various methods of hydrolysis[1]. The procedure of SCHRAM, MOORE AND BIGWOOD[2] in which cystine is oxidized to cysteic acid prior to acid hydrolysis eliminates this objection since cysteic acid is stable during this hydrolysis.

Much difficulty was experienced in this laboratory, however, in the determinations of cysteic acid with ninhydrin reagent[3] following its chromatographic separation on a resin column. This study was undertaken to investigate this difficulty and improve the conditions.

## EXPERIMENTAL AND RESULTS

All the materials to be analyzed for cystine were oxidized with performic acid, according to the procedure of SCHRAM, MOORE AND BIGWOOD[2]. After the removal of excess oxidizing reagents by heating and evaporation on the steam bath, the residue from the oxidation was hydrolyzed by autoclaving with 50 ml of 20% hydrochloric acid for 6 h at 15 lb. pressure. The hydrochloric acid was removed by evaporation on the steam bath. Water added twice, and the sample evaporated almost to dryness after each addition. The autoclaved sample was washed into a 25 ml volumetric flask and made to volume with water. The sample of protein taken for oxidation and hydrolysis was of such a size that a 1- or 2-ml aliquot from the 25 ml-hydrolysate contained approximately 100 or 200 $\mu$g of cystine.

The 1- or 2-ml aliquot was added to a 0.9 × 15 cm column of Dowex-2 X 10 resin in the chloroacetate form. Cysteic acid was eluted with a solution of monochloroacetic acid containing 15 g/l at the rate of 4 ml/h, and a 1-ml fraction was collected in each tube.

Since the fraction collector used was a Time-Flow Technicon machine, tubes on the rack did not always contain exactly the same volume, as would be the case with a Drop-Counter mechanism. Since the eluant was not a buffer, the addition of the same amounts of standard sodium hydroxide did not always produce the pH 5.0 required for the maximum color development with ninhydrin[3]. The values obtained under these conditions were very erratic; some duplicates varied as much as 50%, while others gave good agreement.

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Since a strong sodium hydroxide solution was required to give the desired pH with the approximately 0.16 *N* monochloroacetic acid used as the eluant, it was decided that this alkali solution should have a buffer salt dissolved in it. After several trials, sodium citrate was selected as the most satisfactory. A solution of 1 *N* sodium hydroxide saturated with sodium citrate gave the required pH to the fractions collected in the tubes, but 4 drops were required for each ml eluate collected. The alkali was then changed to 2 *N* sodium hydroxide, saturated with sodium citrate, and 2 drops per tube used. With this procedure, excellent color development was obtained, and the pH after dilution was always very close to 5.0. Recoveries were excellent. Table I shows the values obtained with cysteic acid added to the chromatographic column, eluted with a solution of monochloroacetic acid (15 g/l) and the pH of the tubes adjusted by addition of sodium hydroxide saturated with sodium citrate.

TABLE I

RECOVERY OF CYSTEIC ACID

| *Added* $\gamma$ | *Recovered* $\gamma$ | *Adjusting solution** |
|---|---|---|
| 29.5 | 29.1 | 4 drops *N* NaOH |
| 29.5 | 29.8 | 2 drops 2 *N* NaOH |
| 34.7 | 34.7 | 4 drops *N* NaOH |
| 35.1 | 34.9 | 4 drops *N* NaOH |
| 35.1 | 35.0 | 2 drops 2 *N* NaOH |
| 325.0 | 322.0 | 2 drops 2 *N* NaOH |
| 325.0 | 324.0 | 2 drops 2 *N* NaOH |
| 362.0 | 370.0 | 2 drops 2 *N* NaOH |

* Standard sodium hydroxide solution saturated with sodium citrate to give pH 5.0 in collection tubes, containing 1 ml eluate.

All the tubes used to collect the fractions from the column had been calibrated for 10 ml. After the adjustment to pH 5.0 with the buffered alkali, 2 ml of freshly-prepared ninhydrin solution[3] were added and the tubes heated for 20 min in the steam bath to develop the color. After cooling each tube was diluted to this mark with a solution of 1:1 isopropyl alcohol–water. The color was read in a Beckman Model B Spectrophotometer at 570 m$\mu$ using 1-cm cuvettes. A standard curve had been made using a definite weight of cysteic acid per tube ($\mu$g/ml), and reading at 570 m$\mu$. From this curve, the micrograms of cysteic acid per unit of optical density can be calculated. Using these procedures, the concentration of cysteic acid can be determined directly from the sum of the optical densities of all the tubes containing cysteic acid. It is, therefore, not necessary to correct for color yields relative to leucine nor for evaporation during color development[3].

The addition of 2 drops of 2 *N* sodium hydroxide to each fraction containing approximately 1 ml eluate, gave pH 5.0, which is essential for maximum color development. The use of an unbuffered sodium hydroxide solution with the tubes from a Time-Flow fraction-collector frequently gave less than optimum color development, and, consequently, poor checks for duplicate samples.

As soon as the desired volume of the hydrolysate had been washed onto the column and the funnel containing the eluant had been connected, the column was moved onto the fraction-collector. With the set-up used in this laboratory and a collection rate of 4 ml/h, all the cysteic acid was collected in about 16 tubes, usually between tubes numbered 55–70.

With the materials used, grains, seeds, animal and vegetable proteins, test runs showed there was no added advantage in using a forerun with 0.01 *N* monochloroacetic acid[2]. The use of a solution of monochloroacetic acid containing 15 g/l for the elution of cysteic acid from the column, rather than the 0.1 *N* solution recommended[2], concentrated the cysteic acid in fewer tubes and made the separation sharper.

Blanks for each determination were chosen from the tubes showing very slight color immediately before and after the tubes containing cysteic acid.

The cystine was corrected for the 90% yield on oxidation, according to SCHRAM, MOORE AND BIGWOOD[2].

## SUMMARY

Difficulty was encountered in the determination of cysteic acid with ninhydrin after elution from a resin column, using a Time-Flow fraction-collector. Since the trouble arose from the variable pH obtained on the addition of sodium hydroxide to the eluate, a buffered solution of 2 *N* sodium hydroxide saturated with sodium citrate was used to produce the pH 5.0 required for maximum color development. The calibration for 10 ml of the tubes collecting the eluate, and the use of a standard cysteic acid curve facilitated the calculation of the cysteic acid concentration in the sample.

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# A PREPARATIVE COLUMN FOR THE ION EXCHANGE ISOLATION OF AMINO COMPOUNDS

EUGENE A. TALLEY AND WILLIAM L. PORTER
*Eastern Regional Research Laboratory**, *Philadelphia, Pa.* (*U.S.A.*)

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## INTRODUCTION

The classical methods of MOORE AND STEIN[1] and the automatic modification of SPACKMAN, STEIN AND MOORE[2] for the resolution, identification and quantitative analysis of amino acids in protein hydrolysates have been major contributions to protein chemistry. The application of these methods to natural products, however, has been handicapped by the problem of ascertaining the purity of individual peaks, of identifying unknown compounds, and isolation of sufficient materials for study. Since these analytical ion-exchange methods are micro in scope, other more involved and more tedious procedures must be used to isolate these unknown materials for identification.

HIRS, MOORE AND STEIN[3] reported the use of a 150 × 1.8 cm column for the separation of the mixture resulting from the trypsin hydrolysis of oxidized ribonuclease. This resulted in only a 4-fold increase in capacity and they gave no indication of the comparative resolution obtained with the analytical and preparative columns. PARTRIDGE AND BRIMLEY[4] used three to four coupled columns made from glass pipe for a primary fractionation of the amino acids from protein hydrolysates. Subsequent separations of each fraction were made using new columns, resins and eluants.

This paper reports the results of scaling up the MOORE AND STEIN 100 cm column to the extent of 24 times. By use of this column, sufficient materials for analysis and preparation of derivatives can be obtained with a minimum of effort and change in the ordinary analytical method.

## APPARATUS AND OPERATION

Fig. 1 is a schematic drawing of the scaled-up column. The dimensions were calculated by increasing the cross-sectional area by a factor of 24. The resulting column diameter was 44 mm I.D. and the cross-sectional area was 15.2 cm$^2$. This compares with the 9 mm I.D. analytical column with a cross-sectional area of 0.64 cm$^2$ or a factor of 23.92.

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* Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture, Philadelphia 18, Pa.

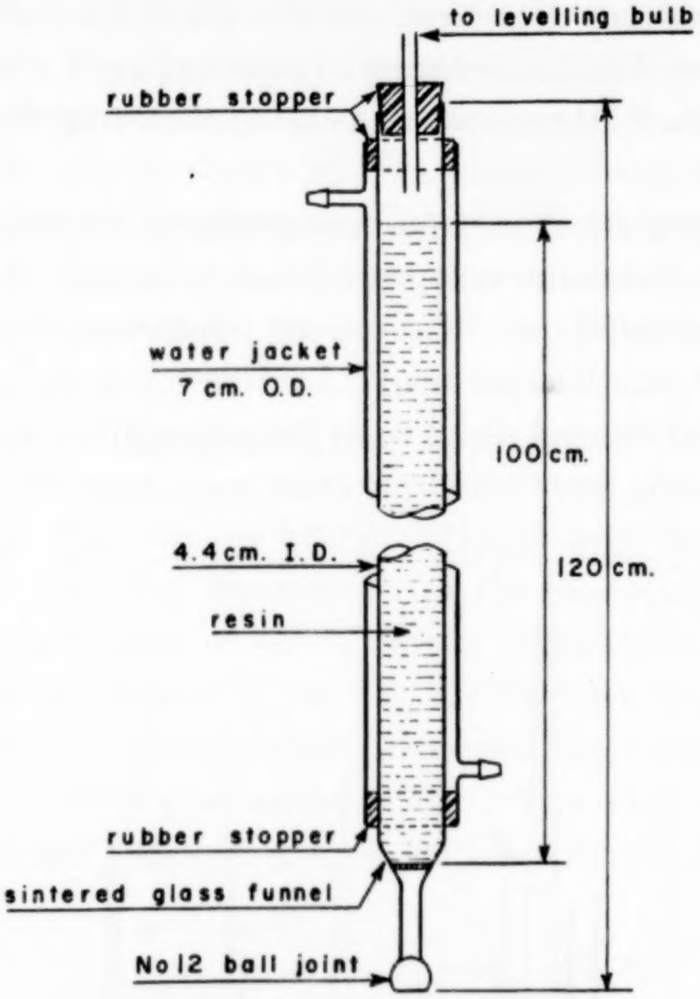

Fig. 1. The scaled-up column.

The resin employed was Dowex-50 X 8*, labeled minus 400 mesh. This preparation was wet sieved through a 200 mesh (U.S.) screen and the fines were removed by backwashing.

The column was constructed by sealing a 60 ml pyrex fritted glass (coarse) funnel to the end of a 115 cm tube, 44 mm I.D. After sealing a No. 12 ball joint to the funnel outlet[5], the overall length of the column was approximately 120 cm. The tube was fitted with rubber stoppers and a surrounding glass tube to jacket the column and control the column temperature.

After mounting the column in an upright position and checking the alignment with a plumb bob, it was filled with resin suspended in pH 3.42 buffer. The resin had been prepared for use according to the treatment of MOORE AND STEIN[1]. About 10–13 cm of buffer was placed in the tube after closing off the ball joint. The resin suspension was added down the side of the tube, by means of a funnel with a bent tip, until about 10–13 cm of column had been poured. The first was allowed to settle until the resin had formed a bed. The tubulation was opened and the buffer allowed to flow through the resin by gravity until no more resin remained in suspension and 10–15 cm of clear buffer remained above the resin bed surface. If the resin surface does not appear to be perfectly flat, the alignment of the column should be checked. Subsequent increments of 10–15 cm of resin suspension were added in this way until the column was about half filled. The remaining portion of the column was poured through a funnel with a

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* Mention of a specific product does not specify recommendation of that product by the Department of Agriculture over any other equal product not mentioned.

U-shaped bend at the bottom to prevent disturbance of the surface of the previously poured and settled layer. This procedure was repeated until a packed column length of 100 cm was obtained. *Extreme care in pouring these columns is required to obtain maximum resolution.*

A 100 × 0.9 cm analytical column was prepared according to the method of MOORE AND STEIN using the same resin preparation as used for the preparative column.

Both columns, mounted on "Technicon" drop-counting fraction collectors, were run at the rate of 8 fractions per hour[5]; fractions of 24 ml and 1 ml were collected at the same flow rate (ml/min/ml resin) from the preparative and analytical columns, respectively. 1 ml aliquots were removed from each fraction from the preparative

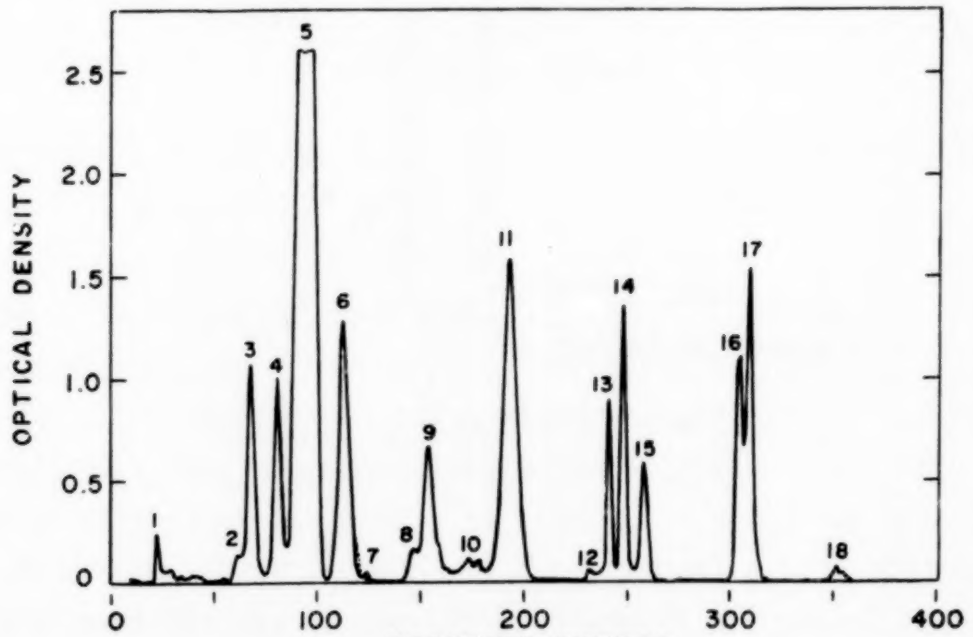

Fig. 2. Preparative column run.

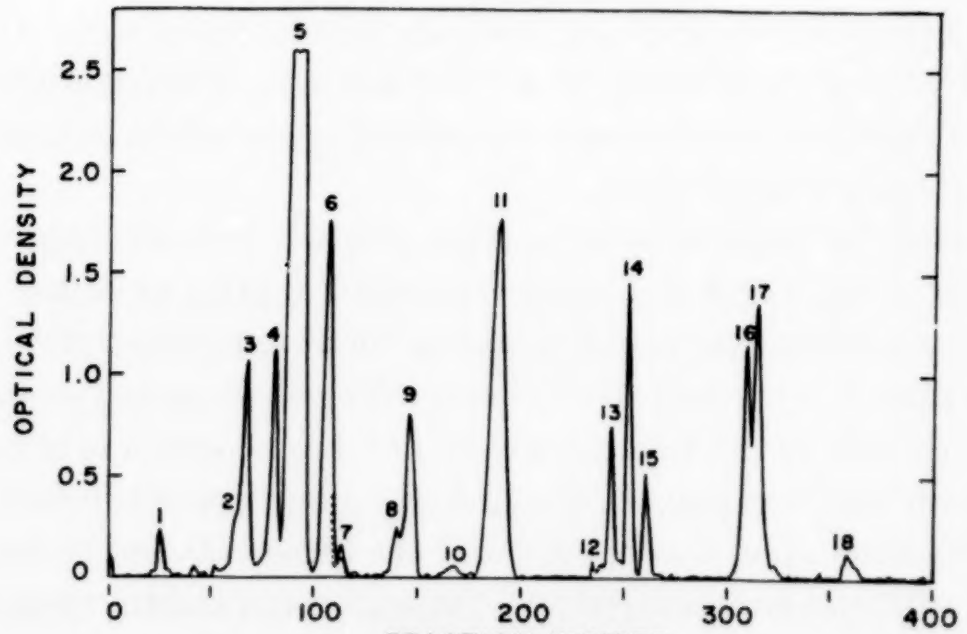

Fig. 3. Analytical column run.

The buffer change (3.42 pH to 4.25 pH) and the first temperature change (37.5° to 50°) were made as fraction 195 filled. The second temperature change (50° to 75°) was made 90 fractions later. Identification of peaks: 1, unknown; 2, unknown; 3, aspartic acid; 4, threonine; 5, asparagine, glutamine and serine; 6, glutamic acid; 7, proline (read at 440 m$\mu$); 8, glycine; 9, $\alpha$-alanine; 10, unknown; 11, valine; 12, unknown; 13, methionine; 14, isoleucine; 15, leucine; 16, tyrosine; 17, phenylalanine; and 18, $\beta$-alanine.

column and placed in the matched test tubes used in the regular analytical procedure. The ninhydrin color was developed and the optical densities were measured in a Beckman Model B spectrophotometer. Using samples of potato extract, in the ratio of 1 to 24 in volume, the results shown in Figs. 2 and 3 were obtained by use of the preparative and the analytical columns respectively. In practice, the remaining 23 ml of each fraction in the individual peaks were combined, desalted by ion exchange, concentrated and crystallized for use in X-ray diffraction, derivatization and analysis.

## RESULTS AND DISCUSSION

The Figs. 2 and 3 show that the resolution obtained with the preparative column was as good or better than that obtained with the analytical column. (The slight variation in effluent volumes may be due in part to variation in fraction size. Similar results were obtained with columns 15 cm long for the basic amino acids.)

In special applications, where certain zones are separated relatively far from other materials, as much as 18 g of amino acid mixture have been resolved on the column. For materials closer together in their elution volumes, lesser amounts must be employed.

## ACKNOWLEDGEMENT

The authors sincerely appreciate the help of Fairie Lyn Carter and Thomas J. Fitzpatrick in building and using these columns.

## SUMMARY

A preparative column is described, which gave a resolution equal to that of an analytical column run under the same conditions.

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# PAPER GEOMETRY AND FLOW VELOCITY IN PAPER CHROMATOGRAPHY*

ARTHUR L. RUOFF AND J. CALVIN GIDDINGS

*Department of Mechanics and Materials, Cornell University, Ithaca, N.Y., and Department of Chemistry, University of Utah, Salt Lake City, Utah (U.S.A.)*

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The velocity of solvent flow in paper chromatography is often an important variable influencing the resolution and indirectly the $R_F$ value of solute zones[1]. The influence of velocity on resolution has not been fully studied in paper chromatography, but theories of the chromatographic process[2] as well as experiments in the basically similar method of gas chromatography[3] show that flow velocity is important. Unlike column chromatography the flow rate cannot be controlled by an applied pressure since in most cases the driving forces for flow are capillary in nature. Three methods are currently available for controlling solvent velocity in a given paper–solvent system at a specified temperature. These are (1) the application of external forces such as centrifugal and gravitational, (2) the addition of surface-active agents to alter the capillary driving forces (this will also influence the chromatographic process to a greater or lesser extent), and (3) the use of different paper geometries such as wick system. The latter method, which is the subject of this communication, has been used especially with circular (horizontal) chromatography with wicks[4]. The general problem has been discussed by MÜLLER *et al.*[5].

An accurate picture of solvent flow in paper is given by assuming that the movement of solvent is governed by the diffusion equations in which the diffusion coefficient is a function of solvent concentration[1]. The equation (Fick's second law) is consequently nonlinear and difficult to solve. If, in addition, one adds the complications of variable geometry, the problem becomes amenable only to extended machine calculations. An approximate theory is presented here which avoids these difficulties, and still gives reasonably quantitative predictions of flow rate.

D'ARCY found that the flow velocity, or flux, in a porous media is proportional to the pressure gradient[6]

$$q = -c\frac{\mathrm{d}P}{\mathrm{d}z} \tag*{1)}$$

where $q$ is the mass flux of solvent per unit width, $W$, of paper strip. The total flux, $q_0$, equals $qW$ and hence

$$q_0 = -cW\frac{\mathrm{d}P}{\mathrm{d}z} \tag{2}$$

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Integrating along the strip from the solvent source at $z_0$ to the solvent front at $z_f$, we have

$$-P = \frac{q_0}{c}\int_{z_0}^{z_f}\frac{\mathrm{d}z}{W(z)} \tag{3}$$

where $P$ is the capillary pressure change from the saturated to the dry paper. Combining this equation with the assumption that the rate of advance of the front is proportional to the flux, *i.e.*, $\mathrm{d}z_f/\mathrm{d}t = bq$, and integrating, we have

$$\int_{z_0}^{z_f}\left[W\int_{z_0}^{z_f}\frac{\mathrm{d}z}{W}\right]\mathrm{d}z_f = \tfrac{1}{2}\varkappa\,(t - t_0) \tag{4}$$

where capillary flow begins at $t_0$, which, in most cases, is arbitrarily set equal to zero. The flow rate coefficient, $\varkappa$, equals $-2bcP$, and is a function of the paper–solvent system. Eqn. (4) is derived on the basis that the flux is constant at every cross section, and that the solvent concentration is uniform throughout the paper. Such an assumption is in error in view of the known concentration gradients[1], but it is necessary in order to obtain mathematical solutions in closed form.

Eqn. (4) has been integrated for the following geometries:

1. Rectangular strips, $z_f^2 = \varkappa t$, the well known parabolic flow equation[5,7,8].
2. Tapered strips with $W = a + mz$, $z_0 = 0$, $m$ either positive or negative,

$$\left(\frac{a}{m} + z_f\right)^2\left[\ln\left(1 + \frac{mz_f}{a}\right) - \tfrac{1}{2}\right] + \tfrac{1}{2}\left(\frac{a}{m}\right)^2 = \varkappa t \tag{5}$$

3. Strip with width discontinuity, $W = a$ for $z = 0$ to $l$, $W = \varepsilon a$ for $z = l$ to $\infty$ ($\varepsilon < 1$). Allowing for the compression of the streamlines a distance $h$ before the discontinuity, we obtain for $z_f > l$

$$\begin{aligned} z_f^2 - g\,(z_f - l) &= \varkappa t \\ g &= 2\,(l - h)\,(1 - \varepsilon) \end{aligned} \tag{6}$$

or, in terms of the dimensionless parameters, $\tau = t\varkappa/l^2$ and $y = z_f/l$

$$y^2 - \frac{g}{l}\,(y - 1) = \tau \tag{7}$$

4. Radial flow in which the solvent source is located a distance $z_0$ from the center of the disc

$$z_f^2 \ln\frac{z_f}{z_0} - \tfrac{1}{2}\,(z_f^2 - z_0^2) = \varkappa t \tag{8}$$

5. Radial flow with a rectangular wick of length $L$ and width $a$ (other wicks such as strings have an effective width proportional to their cross sectional area). Under usual operating conditions we have $L \gg a$ and $z_f \gg a$. With these assumptions an approximate solution to eqn. (4) is

$$z_f^2 = \frac{\varkappa a}{2\pi L}\,t \tag{9}$$

This is parabolic flow in which the flow rate coefficient, $\varkappa_r = \varkappa a/2\pi L$, is significantly less than that for rectangular flow, $\varkappa$. As shown by the form of $\varkappa_r$, the flow rate in radial-wick systems can be very easily controlled by varying the length to width ratio.

Data on the above examples were taken using water on Whatman No. 1 paper at a temperature of 30° ± 0.5°. The use of horizontal flow eliminated gravitational effects. The value of $\varkappa$ was determined using rectangular flow. The experimental and calculated results are shown in Figs. 1–4. The agreement is satisfactory in view of the assumptions made.

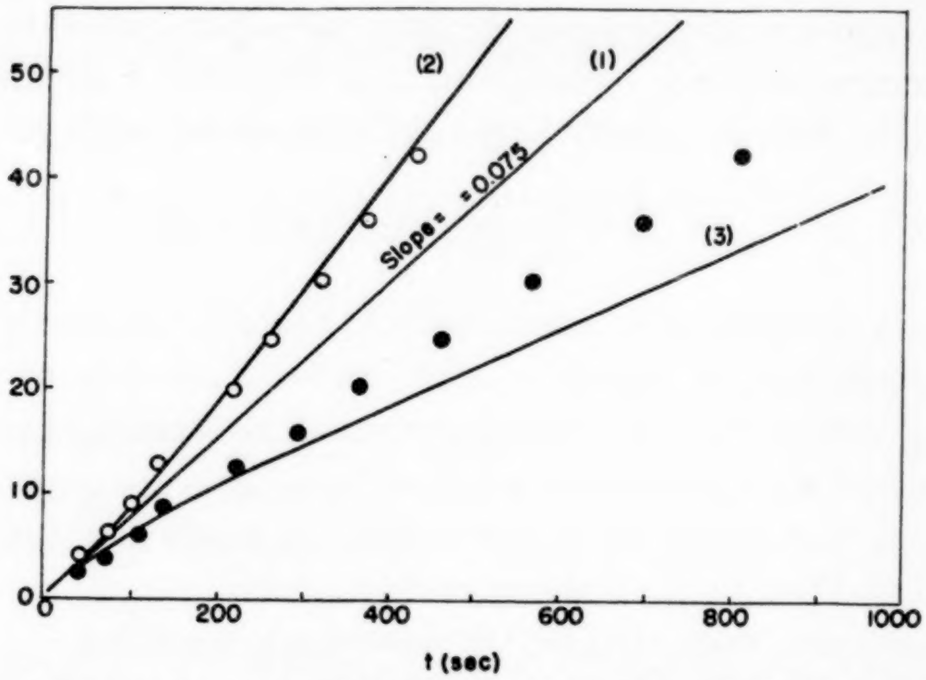

Fig. 1. Flow in tapered strips. (1), rectangular strips; (2), experimental and calculated converging flow ($a$ = 2.0 cm, $m$ = — 0.231); (3), experimental and calculated diverging flow ($a$ = 0.32 cm, $m$ = 0.258).

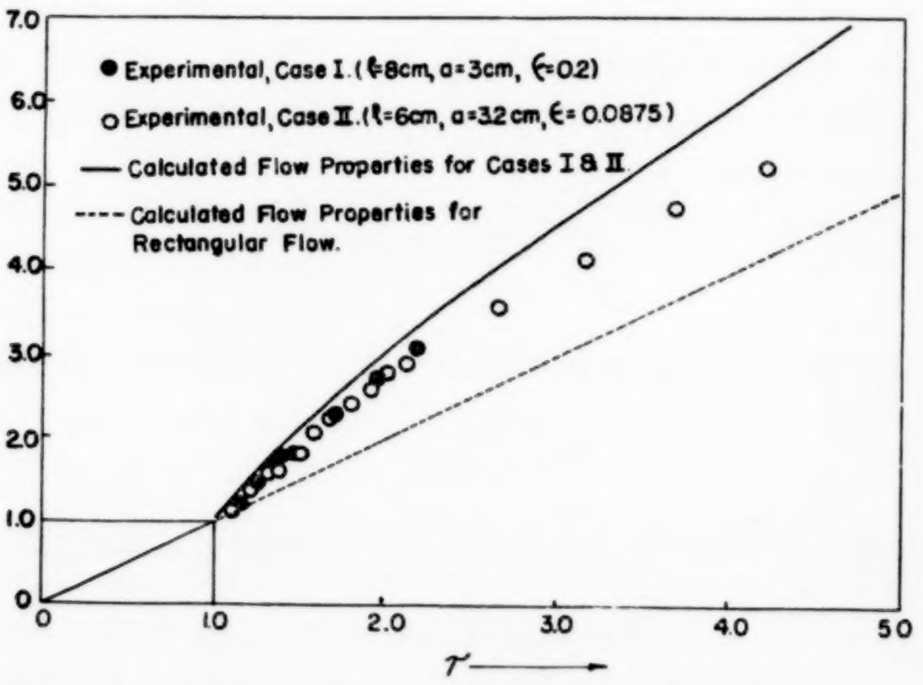

Fig. 2. Flow characteristics with discontinuous width. The width of the shoulder, $a$ (1 — $\varepsilon$)/2 has been used for $h$.

Several points are of particular interest in discussing the results obtained here. First, it can be seen that in all cases of diverging flow (tapered strip with $m > 1$, radial and radial-wick) the front advances more rapidly than predicted while with converging flow (tapered strip with $m < 1$, width discontinuity) the observed velocity is less than calculated. This can be explained in terms of the concentration gradients. Solvent flux is actually divided into two roles, one being the movement of the solvent front as predicted here, and the other being the progressive saturation of the paper, a factor not accounted for in the present theory. In diverging flow, with a relatively wide solvent front, a disproportionate amount goes to the former. This leads to an increased frontal velocity. In converging flow a lesser amount is available for advancing the front, and the decreased velocity results.

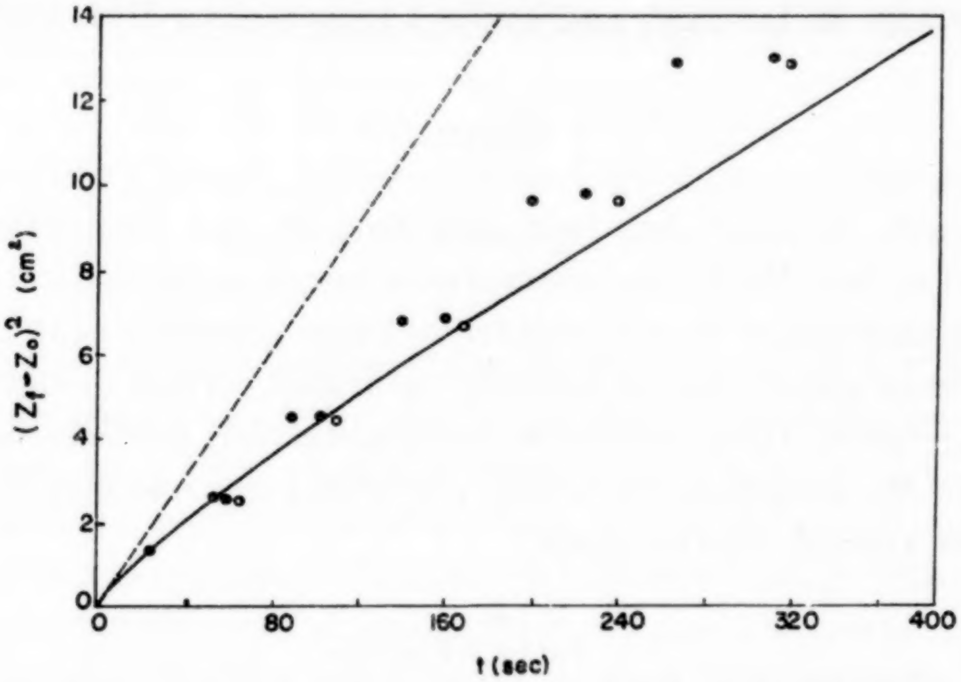

Fig. 3. Radial flow with $z_0 = 0.50$ cm. The solid line is calculated for radial flow and the dotted line for rectangular flow, both with $\varkappa = 0.075$ cm² sec$^{-1}$.

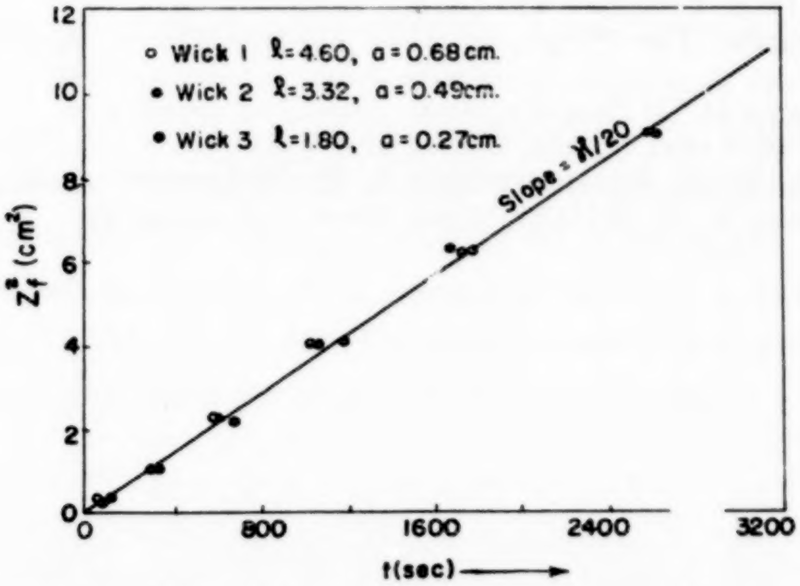

Fig. 4. Radial flow with wick dimensions approximately, $2\pi\, l/a = 42$.

In the case of radial flow with wicks a very satisfactory straight line is obtained plotting $z_f^2$ against time. Furthermore, significant changes in length and width leave the effect of the wick unchanged as long as the length–width ratio is constant. As in other cases of diverging flow, the frontal velocity is larger than predicted. The measured $\varkappa_r$ is $\varkappa/20$ rather than $\varkappa/42$. The use of a large range of wick sizes has shown that the experimentally measured $\varkappa_r$ is approximately $\varkappa a/\pi L$, twice as large as the calculated value, $\varkappa a/2\pi L$. This rule can be effectively used in predicting and controlling flow velocity.

The results obtained here show that with a wick source, the area enclosed by the moving front increases linearly with time. This is verified in the work of HENDRICKSON, BERUEFFY AND MCINTYRE[9], and by LE STRANGE AND MÜLLER[10].

## SUMMARY

The flow of liquids in paper has been described on the assumption that paper is either saturated or dry. With this assumption a simple mathematical treatment can be carried out. It is shown how the use of different paper geometries can be used to control flow velocity. Since paper can be partially saturated certain deviations from experimental data are noted. These deviations are qualitatively predictable. On the basis of the analysis and the experimental results provided here, reasonably accurate predictions of the flow velocity can be made.

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# KINETIC PROCESSES AND ZONE DIFFUSION IN CHROMATOGRAPHY*

J. CALVIN GIDDINGS
*Department of Chemistry, University of Utah, Salt Lake City, Utah (U.S.A.)*

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It has long been realized that kinetic processes involved in the sorption and desorption of solute molecules are in a large part responsible for the smearing of solute zones. The processes that have been especially studied are those in which (1) single step sorption and desorption reactions are important, and (2) diffusion through the stationary or mobile phases is rate controlling. Along with the rapid growth of experimental technique an extended theoretical treatment is needed which will deal in a general way with the complex kinetics which occur in most heterogeneous media such as found in chromatography. An attempt to establish the basis for such a theory is reported elsewhere[1]. We will be concerned here with the discussion of kinetic schemes which more closely approximate real chromatographic systems. Included are the effects of adsorption on heterogeneous surfaces, simultaneous partition and adsorption, adsorption of large molecules, and chemical reactions not directly related to sorption. In some cases simple kinetics are assumed which, although more extensive than found in previous treatments, still are only approximations to some of the very complex systems. Nonetheless this permits us to investigate the gross effects of certain kinds of kinetic processes. The complete theory is briefly outlined which permits the extension to other systems. The general method is applicable to nonlinear kinetics and diffusion-controlled processes, as will be shown.

## THEORY

The theory[1] which permits the evaluation of zone spreading with complex, underlying kinetics is based upon the assumption that the various kinetic steps are proceeding near equilibrium. With only a few exceptions the departure from equilibrium must remain small unless poor resolution is to be tolerated[2].

Each of the various kinetic steps in chromatography can be represented by

$$A_i \underset{k_{ji}}{\overset{k_{ij}}{\rightleftarrows}} A_j \tag{1}$$

where $A_i$ and $A_j$ represent different states of the solute molecule (sorbed, desorbed, hydrogen-bonded etc.). The first order rates of transition between the states are shown

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as $k_{ij}$ and $k_{ji}$. Let the concentration of $i$, referred to a unit volume of the overall column material, be $c_i$, and the total concentration be $c$. The ratio $c_i/c$ is the mole fraction $X_i$. Furthermore each state is characterized by a velocity, $v_i$, relative to the fixed phase. This, of course, is zero if the $i^{\text{th}}$ state is a sorbed state.

The rate of reaction of molecules in the $i^{\text{th}}$ state is obtained by summing over all reaction paths

$$r_i = \sum_j k_{ji}c_j - c_i \sum_j k_{ij} \tag{2}$$

At equilibrium $r_i = 0$ and each concentration is at its equilibrium value $c_i^\star$. In an operating chromatogram the flow of liquid acts to maintain a slight departure from equilibrium. The departure term for the $i^{\text{th}}$ species, $\varepsilon_i$, is defined by

$$c_i = c_i^\star (1 + \varepsilon_i) \tag{3}$$

Substituting this into eqn. (2) and subtracting out the equilibrium terms, which add to zero, we have

$$r_i = \Sigma\, k_{ji}c_j^\star\, \varepsilon_j - c_i^\star\varepsilon_i\ \Sigma\, k_{ij} \tag{4}$$

since at equilibrium forward and reverse rates are equal for each step

$$k_{ji}c_j^\star = k_{ij}c_i^\star \tag{5}$$

we have

$$\frac{r_i}{c_i^\star} = \Sigma\, k_{ij}\varepsilon_j - \varepsilon_i\ \Sigma\, k_{ij} \tag{6}$$

Since the set of equations represented in (6) are linear equations, the $\varepsilon$'s are easily solved for. In order to effect this solution we will assume, as previously stated, that the reactions are proceeding near equilibrium. This assumption is equivalent to saying either that $c_i$ approximately equals $c_i^\star$, or that $\varepsilon_i \ll 1$.

The left hand side of eqn. (6) can be approximated in the following way. The mass balance equation for $i$ is

$$r_i = \frac{\partial c_i}{\partial t} + v_i \frac{\partial c_i}{\partial z} - D_i \frac{\partial^2 c_i}{\partial z^2} \tag{7}$$

where $D_i$ is the diffusion coefficient of $i$ in the longitudinal direction measured by $z$. Since the system is close to equilibrium, $\partial c_i/\partial z$ and $\partial c_i/\partial t$ can be replaced by $\partial c_i^\star/\partial z$ and $\partial c_i^\star/\partial t$. Furthermore it can be shown that the last term is ordinarily negligible[1]. Hence

$$r_i \cong \frac{\partial c_i^\star}{\partial t} + v_i \frac{\partial c_i^\star}{\partial z} \tag{8}$$

Since $c_i^\star$ is a definite fraction, $X_i^\star$, of the total concentration $c$, and since

$$\frac{\partial c}{\partial t} \cong - \bar{u} \frac{\partial c}{\partial z} \tag{9}$$

where $\bar{u}$ is the average velocity of the solute zone, then to a good approximation

$$\frac{r_i}{c_i^\star} = (v_i - \bar{u}) \frac{\partial \ln c}{\partial z} \tag{10}$$

With this evaluation of $r_i/c_i^\star$, the various equations shown in (6) become a set of linear algebraic equations in the various $\varepsilon_i$'s. If there are $n$ states, $i = 1, 2, \cdots n$, we use $n - 1$ of these equations along with the one equation stating that the total concentration adds to $c$. The final equations for the values of $\varepsilon$ are, then

$$\Sigma X_i^\star \varepsilon_i = 0 \qquad (11a)$$

$$(v_i - \bar{u}) \frac{\partial \ln c}{\partial z} = \Sigma k_{ij}\varepsilon_j - \varepsilon_i \Sigma k_{ij} \qquad (11b)$$

Once the values of $\varepsilon$ are known, the zone structure can be related to the $\varepsilon$-dependent flux term. The flux of material through a unit cross section is given by

$$q = \sum_i c_i v_i \qquad (12)$$

Combined with eqn. (3) and the definition of mole fraction, $X_i^\star = c_i^\star/c$, we have

$$q = c\ \Sigma X_i^\star v_i + c\ \Sigma X_i^\star \varepsilon_i v_i \qquad (13)$$

The first summation is merely the average velocity, $\bar{u}$, of the solute zone.

$$q = c\bar{u} + c\ \Sigma X_i^\star \varepsilon_i v_i \qquad (14)$$

The term $c\bar{u}$ represents the flux due to the drift of material along with the solvent.

The significance of the last term, $c\ \Sigma X_i^\star\ \varepsilon_i v_i$, becomes clear with a closer look at the $\varepsilon$ values. From eqns. (11) we see that each $\varepsilon$ is proportional to $\partial \ln c/\partial z$. Since this occurs in each term in the summation it can be factored out and combined with the coefficient $c$ to yield a coefficient $\partial c/\partial z$. Hence the flux is proportional to $\partial c/\partial z$ just as in diffusion processes. Thus it is possible to use an effective diffusion coefficient, $D_c$, in describing the spreading of a zone[2].

Equating the last term in eqn. (14) to $-D_c \partial c/\partial z$, we obtain

$$D_c = \frac{-\ \Sigma X_i^\star \varepsilon_i v_i}{\dfrac{\partial \ln c}{\partial z}} \qquad (15)$$

where the $\varepsilon_i$ values are to be obtained from eqns. (11). It must be remembered that molecular diffusion and "eddy" diffusion also contribute so that the overall coefficient, $D$, is the sum of the three. In those cases where it is advantageous to relate zone structure to the height equivalent to a theoretical plate, $H$, we use the equations

$$H = \frac{2D}{\bar{u}} \quad \text{and} \quad H_c = \frac{2D_c}{\bar{u}} \qquad (16)$$

## HETEROGENEOUS SORPTION

The sorption media encountered in chromatography are rarely expected to be homogeneous. The rates of sorption and desorption would ordinarily vary considerably from one place to another. The 2-site problem in which two different sorption sites with different rate constants are assumed, has been introduced to allow for heterogeneous effects[3,4]. It is possible, however, using the theory just presented, to obtain $D_c$ for the general multi-site sorption problem. This problem allows for the kinetics of sorption on any number of different kinds of sites. The kinetic steps may be written as follows:

$$\begin{array}{c} A_1 \rightleftarrows A_2 \\ A_1 \rightleftarrows A_3 \\ A_1 \rightleftarrows A_4 \\ \vdots \\ A_1 \rightleftarrows A_n \end{array} \tag{17}$$

In this kinetic scheme, $A_1$ represents a solute molecule in the desorbed state, and $A_2$, $A_3$, $\cdots A_n$ represent different sorption states of the molecule. There are $2n - 2$ different rate constants of the form $k_{ij}$.

Eqns. (11a) and (11b) can be applied to evaluate the $\varepsilon$'s. For the $n - 1$ equations of the form (11b), it is convenient to choose those for which $i = 2, 3, \cdots n$. Each $v_i$ equals zero for this range of $i$'s. The $n$ simultaneous equations are

$$\begin{array}{l} g = k_{21}\varepsilon_1 - k_{21}\varepsilon_2 \\ g = k_{31}\varepsilon_1 \qquad\qquad - k_{31}\varepsilon_3 \\ \vdots \\ g = k_{n1}\varepsilon_1 \qquad\qquad\qquad\qquad - k_{n1}\varepsilon_n \\ 0 = X_1^\star\varepsilon_1 + X_2^\star\varepsilon_2 + X_3^\star\varepsilon_3 \cdots + X_n^\star\varepsilon_n \end{array} \tag{18}$$

where $g$ has been substituted for $-\bar{u}\,\partial \ln c/\partial z$. Eqn. 15 tells us which of the $\varepsilon$'s must be evaluated. Since $v_i$ is in each term of the summation, and $v_i = 0$ for all but $i = 1$, it is only necessary to obtain $\varepsilon_1$. Cramer's rule can be applied to this problem, and yields the following ratio of determinants.

$$\varepsilon_1 = \frac{\begin{vmatrix} g & -k_{21} & 0 & 0 & \cdots & 0 \\ g & 0 & -k_{31} & 0 & & 0 \\ \vdots & & & & & \vdots \\ g & 0 & 0 & 0 & & -k_{n1} \\ 0 & X_2^\star & X_3^\star & X_4^\star & \cdots & -X_n^\star \end{vmatrix}}{\begin{vmatrix} k_{21} & -k_{21} & 0 & 0 & \cdots & 0 \\ k_{31} & 0 & -k_{31} & 0 & & 0 \\ \vdots & & & & & \vdots \\ k_{n1} & 0 & 0 & 0 & & -k_{n1} \\ X_1^\star & X_2^\star & X_3^\star & X_4^\star & \cdots & X_n^\star \end{vmatrix}} \tag{19}$$

This can be reduced by the standard methods to yield

$$\varepsilon_1 = g \sum_{i=2}^{n} \frac{X_i^\star}{k_{i1}} \tag{20}$$

Substituting this back into eqn. (15) we obtain

$$D_c = \bar{u}^2 \, \Sigma \, \frac{X_i^\star}{k_{i1}} \tag{21}$$

which is the effective coefficient of diffusion due to the kinetics. This result will now be expressed in a form directly related to desorption kinetic measurements.

A convenient parameter in the study of desorption kinetics is the average time required for desorption, $\bar{t}_d$. This is equal to the mean sorption life time of a collection of molecules sorbed at equilibrium and which can be irreversibly desorbed, *i.e.*, removed from further sorption after desorption. An expression for $\bar{t}_d$ is

$$\bar{t}_d = \frac{\int_0^\infty \Sigma X_i \, \mathrm{d}t}{1 - R} \tag{22}$$

where $R$ is the fraction of molecules initially desorbed (in the mobile phase), and $X_i$ is the mole fraction in the $i^{\text{th}}$ sorbed state at the time $t$. At $t = 0$, $X_i = X_i^\star$ for all $i$. Since the desorption is irreversible, each $X_i$ follows the first order rate law

$$X_i = X_i^\star \, \mathrm{e}^{-k_{i1}t} \tag{23}$$

Substituting this back into eqn. (22) and evaluating the integral, we obtain

$$\bar{t}_d = \Sigma \frac{X_i^\star}{k_{j1}} / (1 - R) \tag{24}$$

where all summations run from $i = 2$ to $i = n$. This in turn can be substituted into eqn. (21), which yields

$$D_c = \bar{u}^2 \, (1 - R) \bar{t}_d \tag{25}$$

An equivalent form is obtained when $Rv$ is substituted for $\bar{u}$. The quantity $v$ is the average stream velocity of the mobile phase while $R$ is the ratio of zone to stream velocity.

$$D_c = R^2 v^2 \, (1 - R) \, \bar{t}_d \tag{26}$$

Eqns. (25) and (26) are interesting since the only kinetic quantity appearing is the mean desorption time, $\bar{t}_d$. This result permits a more realistic interpretation of chromatographic experiments since it is no longer necessary to account for the entire kinetic effect by one or two hypothetical reactions. It would be desirable, however, to isolate the various rate contributions to $\bar{t}_d$. This would probably require the combined data from several fields of study, including chromatography. It appears that chromatography might be useful in studying desorption phenomena as well as several other reaction rate processes in porous media.

The above equations for $D_c$ are, of course, limited to kinetics near equilibrium. If any of the desorption steps are significantly slow, then the influence of that step cannot be included in eqns. (25) and (26). The procedure then is to account for all the steps possible by means of eqns. (25) or (26), and then include the effect of the nonequilibrium step or steps separately. This can be done if the nonequilibrium steps are sufficiently simple to apply the exact theories of chromatography. Some promise has been found by this method in interpreting double spots in paper chromatography[5].

### CONSECUTIVE REACTIONS

A large number of kinetic processes in chromatography involve a series of consecutive reactions. The following kinetic scheme roughly approximates many of these processes

$$\mathrm{A}_1 \underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}} \mathrm{A}_2 \underset{k_{32}}{\overset{k_{23}}{\rightleftarrows}} \mathrm{A}_3 \tag{27}$$

Let the flow velocities be $v_1$, $v_2$ and $v_3$ respectively. Any one of these may again be zero if the state is a sorbed one. Eqns. (11a) and (11b) can be applied to the above kinetics with the result

$$g_1 = -k_{12}\varepsilon_1 + k_{12}\varepsilon_2 \tag{28a}$$

$$g_3 = k_{32}\varepsilon_2 - k_{32}\varepsilon_3 \tag{28b}$$

$$0 = X_1^\star\varepsilon_1 + X_2^\star\varepsilon_2 + X_3^\star\varepsilon_3 \tag{28c}$$

where

$$g_1 = (v_1 - \bar{u})\frac{\partial \ln c}{\partial z}, \qquad g_3 = (v_3 - \bar{u})\frac{\partial \ln c}{\partial z}, \quad \text{etc.} \tag{29}$$

Applying Cramer's rule we obtain $\varepsilon_1$ and $\varepsilon_3$

$$\varepsilon_1 = -\frac{g_1}{k_{12}} + \frac{g_1}{k_{12}}X_1^\star + \frac{g_3}{k_{32}}X_3^\star \tag{30a}$$

$$\varepsilon_3 = -\frac{g_3}{k_{32}} + \frac{g_3}{k_{32}}X_3^\star + \frac{g_1}{k_{12}}X_1^\star \tag{30b}$$

These can be substituted into eqn. (15) for $D_c$. First, however, we obtain the $\varepsilon_2$ term in the summation directly from eqn. (28c).

$$-X_2^\star\varepsilon_2 = X_1^\star\varepsilon_1 + X_3^\star\varepsilon_3 \tag{31}$$

The result is

$$\frac{\partial \ln c}{\partial z}D_c = (v_2 - v_1)\,X_1^\star\varepsilon_1 + (v_2 - v_3)\,X_3^\star\varepsilon_3 \tag{32}$$

Using eqns. (30) for $\varepsilon_1$ and $\varepsilon_2$, and an expression for the mean velocity

$$\bar{u} = X_1^\star v_1 + X_2^\star v_2 + X_3^\star v_3 \tag{33}$$

we obtain

$$D_c = \frac{X_1^\star\,(v_1 - \bar{u})^2}{k_{12}} + \frac{X_3^\star\,(v_3 - \bar{u})^2}{k_{32}} \tag{34}$$

It will be noticed that in the special case where $v_1 = v_3 = 0$ we have the 2-site sorption problem with $A_2$ representing the mobile species. Under these circumstances eqn. (34) reduces to eqn. (21).

Eqn. (34) may be used in describing reactions that occur in either the mobile or stationary phase independent of the phase transfer process. The additional reaction might be an association reaction with a species present in one of the phases. It is necessary that the reaction remains first order in the solute concentration, although the overall order of the reaction may be different.

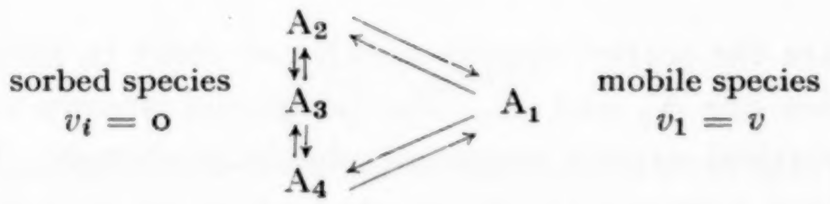
The occurrence of some adsorption in a process that is primarily partition has concerned workers in both gas and paper chromatography. This example is a valid application of eqn. (34) as long as the isotherm remains linear. The use of a single reaction step to describe the partition-diffusion process must be considered as an approximation. Let $A_1$ be the mobile phase species, $A_2$ the absorbed, and $A_3$ the adsorbed species. The respective velocities are $v_1 = v$, $v_2 = v_3 = 0$. Furthermore $X_1^\star = R$ and $X_2^\star + X_3^\star = 1 - R$. With these values $D_c$ becomes

$$D_c = \frac{R\,(1 - R)^2\,v^2}{k_{12}} + \frac{X_3^\star R^2 v^2}{k_{32}} \tag{35}$$

The first term on the right is the one obtained when no adsorption is occurring. The second term is the contribution of adsorption. It is seen that this increases with the amount, $X_3^{\star}$, adsorbed at equilibrium, and furthermore is generally significant when the adsorption process is slow. As a rough rule for cases with intermediate $R$ values, the contributions to $D_c$ will be in proportion to the time of reaction, or inversely proportional to the reaction rate constants.

## LARGE MOLECULES

The sorption of large molecules is by itself a complex phenomenon. The sorption process consists of a series of steps in which one segment attaches itself to the sorbent followed by the sorption of additional segments. Since the general problem is exceedingly complex[6], we will show the nature of a simple prototype involving bi-functional sorption. This is a case in which only two parts of a molecule are active in attaching to the surface (molecules such as dicarboxylic acids with two polar ends would have kinetics approaching that of the prototype). The following kinetic scheme describes the situation.



(36)

The detached molecule $A_1$ becomes $A_2$ if it anchors by means of one of its active groups, and $A_4$ if it anchors by means of the other. The two active groups are not necessarily the same. The molecule can then desorb back to $A_1$ or complete the sorption of the other active group to form the completely sorbed molecule $A_3$.

The methods used in previous sections can be applied to the present example to yield the following $D_c$

$$D_c = \frac{Rv^2 \begin{bmatrix} (1 - R)\,(k_{23}k_{40}X_2^{\star} + k_{20}k_{40}X_3^{\star} + k_{43}k_{20}X_4^{\star}) \\ + R\,(k_{12}\,(k_{23} - k_{43})\,X_4^{\star} + k_{14}\,(k_{43} - k_{23})\,X_2^{\star} - k_{12}k_{40}X_3^{\star} - k_{14}k_{20}X_3^{\star}) \end{bmatrix}}{k_{12}k_{23}k_{40} + k_{14}k_{43}k_{20}} \tag{37}$$

where $k_{20}$ and $k_{40}$ have been substituted for $(k_{21} + k_{23})$ and $(k_{41} + k_{43})$, respectively.

Eqn. (37) simplifies considerably under certain limiting circumstances. Consider for example a molecule in which the intermediates $A_2$ and $A_4$ have a short lifetime. Such would occur especially when the activation energy for further sorption is small. Under these circumstances $X_2^{\star}$ and $X_4^{\star}$ become negligible compared to $X_3^{\star}$ while $k_{20} \gg k_{12}$ and $k_{40} \gg k_{14}$. Also $X_3^{\star}$ becomes equal to $(1 - R)$. When these assumptions are used in eqn. (37) we have

$$D_c = \frac{R\,(1 - R)^2\,v^2}{k_{12}f_2 + k_{14}f_4} \tag{38}$$

where $f_2 = k_{23}/k_{20}$ is the fraction of times that a molecule in the form $A_2$ proceeds to the completely sorbed state $A_3$. A like interpretation holds for $f_4$.

## CHEMICAL CHANGES

Chemical changes in a chromatographic column lead to a variety of phenomena depending upon the nature and rates of the reactions. If the rates of chemical change are slow, large nonequilibrium effects are observed. These effects include the formation of double and triple zones, and also tailing[5]. If the reactions proceed rapidly enough to effect a large number of chemical changes during the running time of the experiment, the departure from equilibrium will be small, and the present procedure valid. In addition to predicting chromatographic performance from reaction kinetics, it is suggested that the inverse process of obtaining reaction rate data from chromatography might be fruitful.

One type of chemical change can be given as follows

$$\begin{tikzcd}
A_1 \arrow[r, leftrightarrow] \arrow[d, leftrightarrow] & A_2 \arrow[d, leftrightarrow] & \text{chemical change} \\
A_4 \arrow[r, leftrightarrow] & A_3 &
\end{tikzcd} \tag{39}$$

$$v_1 = v_4 = 0 \qquad v_2 = v_3 = v$$

where $A_1$ and $A_4$ are the sorbed species which can react to form one another. The mobile phase species are $A_2$ and $A_3$. The horizontal arrows represent the phase changes and the vertical arrows represent chemical changes. This scheme would apply to isomerization reactions (*cis-trans*, etc.) which occur in both the mobile and stationary phases. It would also be applicable to a solute that combines with and dissociates from some species at constant concentration within the column.

The evaluation of $D_c$ for the above scheme involves detailed manipulations too long to reproduce here. The result is

$$D_c = \frac{v^2 \left\{ \begin{array}{l} (1 - R) \left[ \begin{array}{l} X_1^{\star} X_2^{\star} k_{14} (k_{30} + k_{23}) + X_1^{\star} X_3^{\star} (k_{14} (k_{20} + k_{32}) - k_{12} k_{34}) \\ + X_2^{\star} X_4^{\star} k_{10} (k_{30} + k_{23}) + X_3^{\star} X_4^{\star} (k_{10} (k_{20} + k_{32}) - k_{12} k_{21}) \end{array} \right] \\ \quad + R [k_{23} k_{34} X_1^{\star} X_2^{\star} + k_{34} k_{20} X_1^{\star} X_3^{\star} - k_{21} k_{30} X_2^{\star} X_4^{\star} - k_{21} k_{32} X_3^{\star} X_4^{\star}] \end{array} \right\}}{k_{14} k_{21} k_{30} + k_{23} k_{34} k_{10}} \tag{40}$$

where, again, $k_{30} = k_{32} + k_{34}$, etc. This equation can be simplified by making the following approximations. We will assume that the phase change reactions are much more rapid than the chemical reactions, *i.e.*, $k_{12}$, $k_{21}$, $k_{43}$, $k_{34} \gg k_{14}$, $k_{41}$, $k_{23}$, $k_{32}$. With this approximation we obtain

$$D_c = \frac{v^2 (X_1^{\star} X_3^{\star} - X_2^{\star} X_4^{\star})^2}{k_{14} X_1^{\star} + k_{23} X_2^{\star}} + \frac{v^2 X_1^{\star} X_2^{\star}}{(X_1^{\star} + X_2^{\star})(k_{12} + k_{21})} + \frac{v^2 X_3^{\star} X_4^{\star}}{(X_3^{\star} + X_4^{\star})(k_{34} + k_{43})} \tag{41}$$

The terms in this equation can be interpreted as follows. The second term is the contribution due to the phase change of the chemical species ($A_1$, $A_2$), and as such contains only $k_{12}$ and $k_{21}$. The third term has an analogous meaning for ($A_3$, $A_4$). The first term accounts for the effects of chemical change. This term can alternately be derived by assuming that each of the two chemical species is moving with its own particular velocity characterized by its own $R$ value (it must be assumed, also, that the sorption or partitioning process is near equilibrium). This derivation proceeds from the fact that any two interconverting species moving at different velocities gives rise to a diffusion effect. In fact, the simplest kinetic picture of chromatography is based on this fact: the sorbed and desorbed molecules are moving at different downstream velocities, and are, of course, interconverting.

Examination of the first term in eqn. (41) shows that a zero value is acquired when $(X_1^{\star}X_3^{\star} - X_2^{\star}X_4^{\star}) = 0$. This is found when the separate $R$ values $(X_2^{\star}/(X_1^{\star} + X_2^{\star})$ and $X_3^{\star}/(X_3^{\star} + X_4^{\star}))$ are equal, or when the separation factor is unity. The importance of the chemical change term, then, depends on two factors; the difference in $R$ values appearing in the numerator and the reaction rate constants appearing in the denominator.

It is expected that gas chromatography (especially with capillary columns) will prove a useful medium to exhibit the chemical change term, even with fairly rapid reaction rates. This is a result of the small contribution of the second and third terms of eqn. (41) as illustrated by the fact that the order of $10^6$ theoretical plates can now be obtained on capillary columns.

## CONCLUSIONS

While the above examples have been concerned primarily with rate processes involving discrete kinetic steps, the nonequilibrium method is also applicable to diffusion controlled processes. These processes lead to a nonequilibrium in the form of lateral concentration gradients. Since the departure from equilibrium is generally small, the same approximations can be used in the mass conservation equations. The rate of reaction, $r_i$, becomes the rate of accumulation due to the lateral diffusion. Both particle-wide (referring to the particles of the porous media) and tube-wide nonequilibrium can exist in a chromatographic column. An example of the latter is the concentration gradient existing laterally across a coiled column[7]. Particle-wide nonequilibrium will be the subject of a later communication.

The nonequilibrium method can also be used in the description of nonlinear kinetics. While the rate equations (2) are nonlinear, the situation is still tractable as long as $\varepsilon_i \ll 1$. With this condition applying, we can make approximations of the following kind

$$\begin{aligned} c_i^2 &= c_i^{\star 2}(1 + 2\varepsilon_i) \\ c_i c_j &= c_i^{\star} c_j^{\star}(1 + \varepsilon_i + \varepsilon_j) \end{aligned} \tag{42}$$

where terms the order of $\varepsilon^2$ have been discarded. When these expressions are substituted back into the rate equations they are still linear in the $\varepsilon$'s.

The final results obtained for the various examples have been presented in terms of an effective diffusion coefficient, $D_e$. This can be written in terms of plate height by eqn. (16). The concentration profiles observed in chromatography, which have not been derived here, are obtained by applying Fick's laws of diffusion to the entering zone. The final profile differs with the nature of the original zone. Since the mathematics of diffusion is such a highly developed discipline, it is felt that concentration profiles can be effectively derived by its use rather than by means of a separate mathematical derivation using the plate-height concept. The use of diffusion in describing zone structure has been shown by GLUECKAUF[8]. One case of zone structure is found so universally that its relation to the present treatment should be mentioned. When the solute is started as a narrow zone and there are no outside gradients, diffusion leads to a "Gaussian" peak in which the root-mean-square deviation, $\sigma$, is equal to $\sqrt{2Dt}$.

The methods used to derive $D_c$ in this paper are not limited to chromatography, but can be applied to a large class of differential migration problems in which rate processes are occurring. Electrophoresis and sedimentation are examples in which zone diffusion results from kinetics processes[9].

## TABLE OF SYMBOLS

| | |
|---|---|
| $A_i$ | chemical species of the $i^{th}$ type |
| $c$ | total solute concentration, referred to unit volume of overall chromatogram |
| $c_i$ | concentration of species $i$ |
| $c_i^*$ | equilibrium concentration of species $i$ |
| $D$ | total diffusion coefficient in the direction of flow |
| $D_c$ | diffusion coefficient in flow direction due to nonequilibrium effects |
| $D_i$ | diffusion coefficient in flow direction of species $i$ |
| $\varepsilon_i$ | equilibrium departure term |
| $f_2$, $f_4$ | defined following eqn. (38) |
| $g_i$ | abbreviation for $(v_i - \bar{u})\ \partial \ln c/\partial z$ |
| $H$ | total plate height |
| $H_c$ | plate height due to nonequilibrium effects |
| $k_{ij}$ | rate constant for the $A_i$ to $A_j$ transistion |
| $n$ | the number of reacting species |
| $q$ | solute flux in flow direction |
| $R$ | equilibrium fraction of molecules in the mobile phase |
| $r_i$ | rate of accumulation of $i$ due to the kinetic processes |
| $t$ | time |
| $\bar{t}_d$ | mean desorption time of sorbed molecules |
| $\bar{u}$ | mean velocity of solute zone |
| $v_i$ | velocity of species $i$ in flow direction |
| $X_i$ | mole fraction of solute in the $i^{th}$ form |
| $X_i^*$ | equilibrium mole fraction of $i$ |

## SUMMARY

Equations have been obtained which give the effective diffusion coefficient or plate height due to kinetic processes. It is assumed that the departure from equilibrium is only slight corresponding to the situation usually found in chromatography. The theory is applied to sorption on heterogeneous media, consecutive reactions, the chromatography of large molecules, and the occurrence of chemical change simultaneous with chromatography. The extension of the theory to diffusion-controlled processes and nonlinear kinetics is discussed. The applicability to other differential migration methods is noted.

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# HEATS OF PREFERENTIAL SORPTION FROM LIQUID MIXTURES

A. GROSZEK

*The British Petroleum Company Limited, Petroleum Division,*
*BP Research Centre, Sunbury-on-Thames, Mx. (Great Britain)*

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## INTRODUCTION

It is well known that adsorption of liquids on dry surfaces of solids is accompanied by the evolution of heat. Such heat effects measure the changes in surface energy of the solids and adsorbate produced in the process of adsorption.

Similar heat effects were found to take place when one liquid displaced another as an adsorbed layer on a solid, or as a sorbed liquid. Where the displacing liquid is much more strongly sorbed or adsorbed than that displaced, the heat effect is considerable even when the displacing liquid is introduced as a dilute solution in the liquid being displaced. Such displacement phenomena when applied to adsorption have been referred to in the literature as processes of preferential adsorption[1]. In this paper the sorption and adsorption phenomena are both referred to under the general name of preferential sorption.

The process of preferential adsorption is of importance in the field of solid–liquid chromatography, where it forms a basis for the separations of mixtures into fractions of different chemical and physical properties[1]. The type of separation achieved by a given adsorbent can be predicted on the basis of the determinations of the heat of preferential adsorption. Such predictions should be more realistic than those based on determinations of the heats of wetting of dry adsorbents by different constituents of a mixture.

This paper describes determinations of the heats of preferential sorption by a specially constructed calorimeter. The results presented illustrate application of the calorimeter to determination of the heats produced on a number of different adsorbents including an ion-exchange resin and a metal soap.

## EXPERIMENTAL

### *Apparatus and procedure*

The apparatus used is represented in Fig. 1. The apparatus has been described previously[2] and its prototype described in this Journal[3]. It consists essentially of a cell filled with an adsorbent, thermocouples placed in a direct contact with the adsorbent, and a suitable jacketing arrangement. The cell is kept at room temperature, *i.e.* 20° ± 2°. The lagging surrounding the cell is sufficient to eliminate any sudden changes of temperature inside the cell.

In the cell are placed 15 thermocouples connected in series and composed of 32 SWG copper and constantan wires. The hot junctions of the thermocouples are situated immediately above the plug and are sealed, by means of Araldite D cement, into the wall of the cell. The ends of the thermocouples are only slightly protruding from the wall and are in direct contact with the powdered solid. The wires from the cold junctions pass out of the glass jacket through holes in the rubber bung to the outside where the cold junctions are kept at the temperature of the surrounding medium. The output from the thermocouples is fed into a potentiometric recorder of 500 $\mu$V full scale deflection. A coil of nichrome wire possessing a resistance of 10 $\Omega$ is situated in the centre of the cell. The coil is used to reproduce the heat effects taking place during adsorptions, by passing through it a small current from a source of constant known voltage, for a measured length of time.

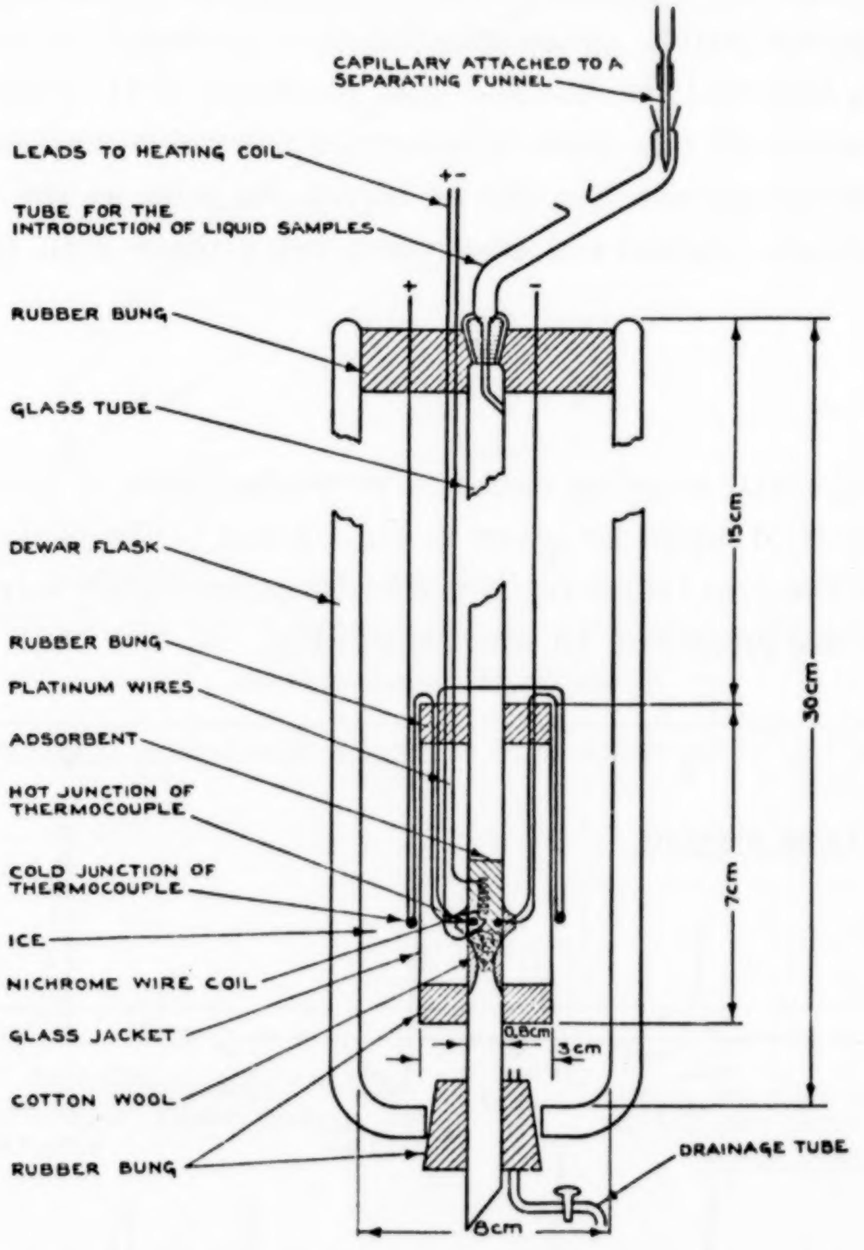
CAPILLARY ATTACHED TO A SEPARATING FUNNEL
LEADS TO HEATING COIL
TUBE FOR THE INTRODUCTION OF LIQUID SAMPLES
RUBBER BUNG
GLASS TUBE
DEWAR FLASK
RUBBER BUNG
PLATINUM WIRES
ADSORBENT
HOT JUNCTION OF THERMOCOUPLE
COLD JUNCTION OF THERMOCOUPLE
ICE
NICHROME WIRE COIL
GLASS JACKET
COTTON WOOL
RUBBER BUNG
DRAINAGE TUBE
+ -
+
-
15cm
30cm
7cm
0,8cm
3cm
8cm

Fig. 1. Calorimeter for determination of heats of preferential sorption.

A glass tube is connected directly to the cell and extends to a special attachment which is used for introducing small quantities of the substance under examination from a micrometer syringe into a carrier liquid flowing continuously through the cell. The carrier liquid is contained in a separating funnel and its flow is regulated by a capillary jet attached to the end of the funnel. The flow is usually between 0.3 and 0.5 ml/min.

Initially, when the carrier liquid comes into contact with the dry adsorbent in the cell, a temperature rise is produced. From this rise it is possible to obtain, if it is so desired, the heat of adsorption of the carrier liquid on the adsorbent saturated with air. After approximately 30–40 min the heat produced by wetting of the dry adsorbent is dissipated and the hot and cold junctions of the thermocouples are at the same temperature. At this point a measured amount of another liquid, or solid in solution, is introduced into the carrier liquid. As soon as the injected material reaches the adsorbent, preferential adsorption takes place with the accompanying evolution of heat.

### *Results*

The heats of preferential sorption obtained on replacement of a carrier liquid by a more strongly adsorbed liquid are given in Fig. 2a and b; the peaks shown are scaled down to about half the size of those registered by the potentiometric recorder. Two series of determinations are presented. In one series (Fig. 2a) the heat effects are due to preferential sorption of liquids on lithium stearate from benzene which was the carrier liquid. The benzene peak in Fig. 2a was produced by replacement of *n*-heptane by benzene. In the preferential adsorption on carbon black (Fig. 2b), the heat effects are due to replacement of *n*-heptane from the adsorbent.

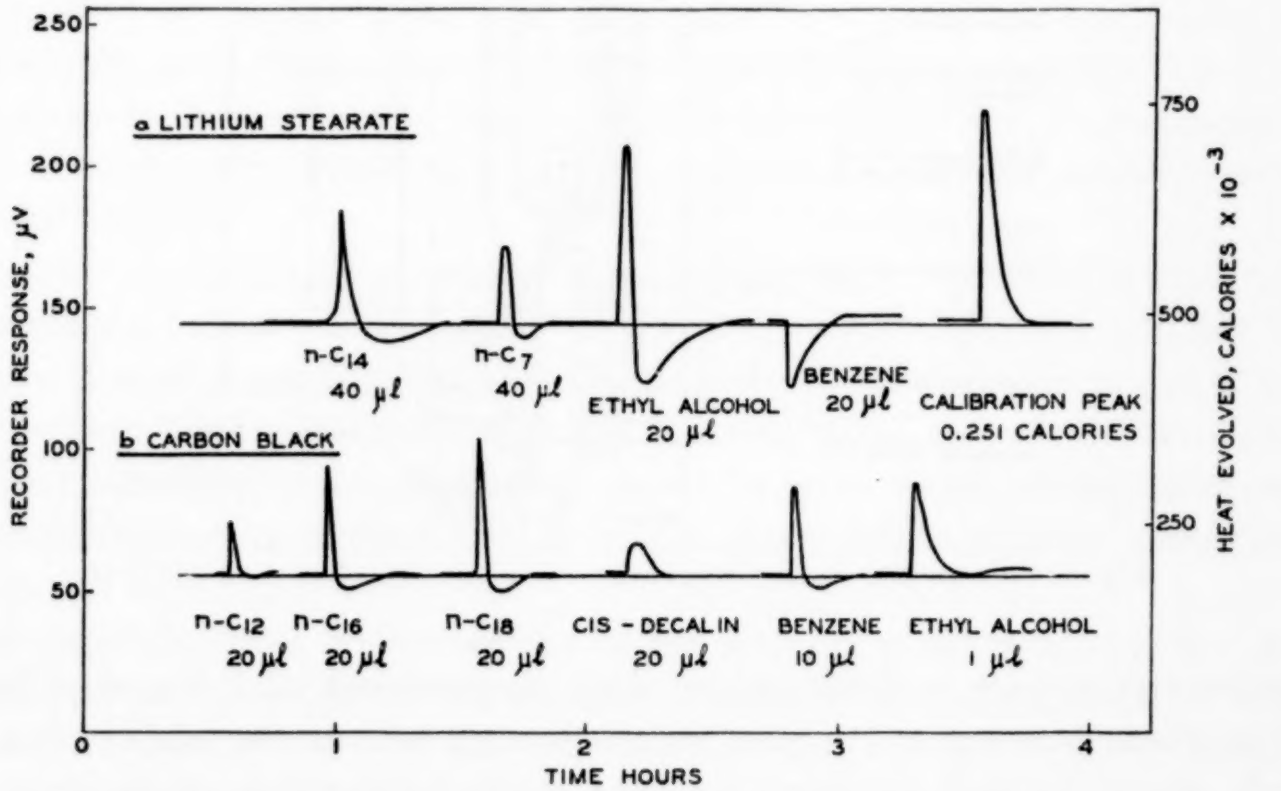

Fig. 2. Heats of preferential sorption registered by a recording potentiometer.

In both series the carrier liquid was flowing through the adsorbents at the rate of 0.4 ml/min.

In Figs. 3 and 4 the heats produced by the preferential sorption by the two solids

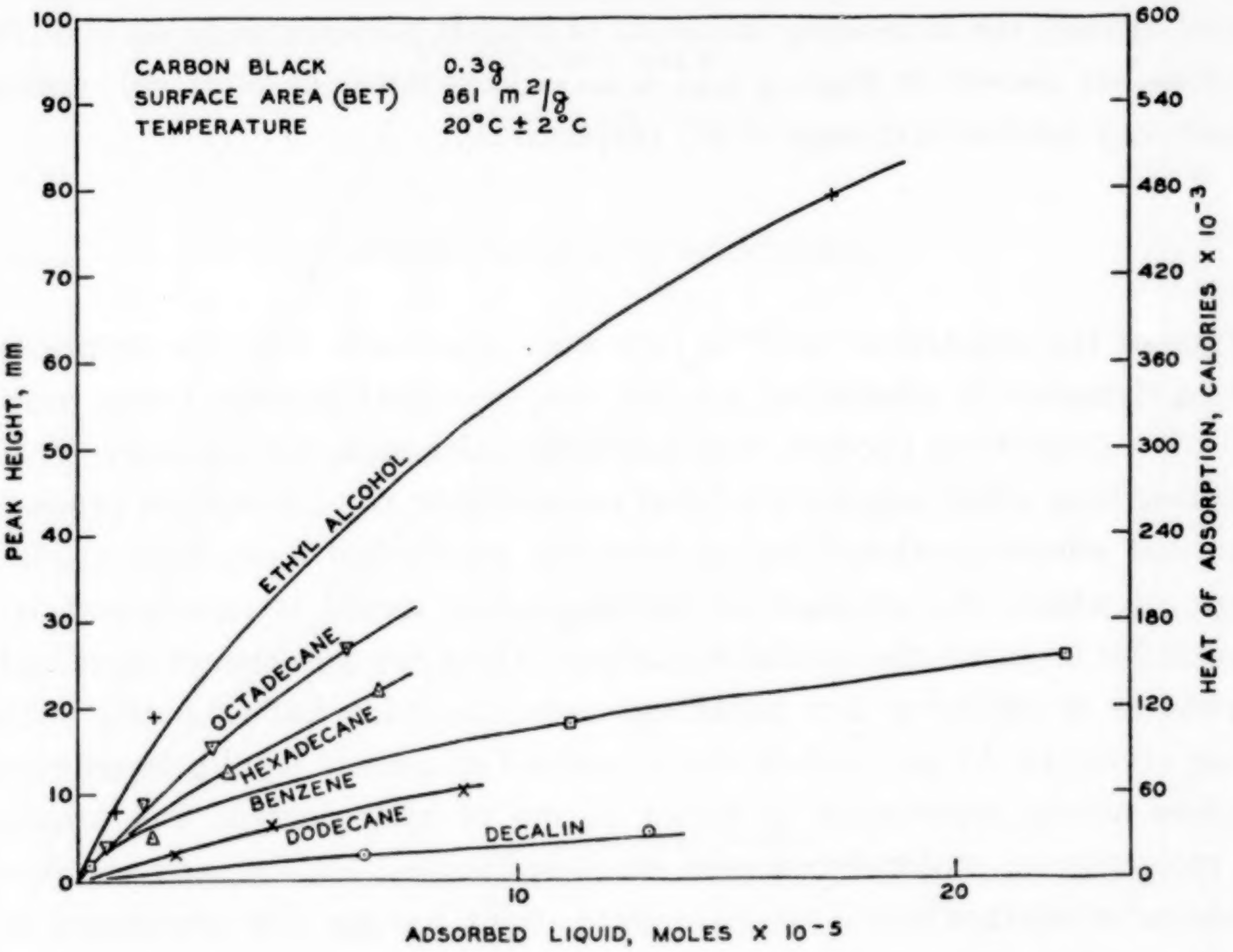

Fig. 3. Heats of preferential adsorption from *n*-heptane on carbon black.

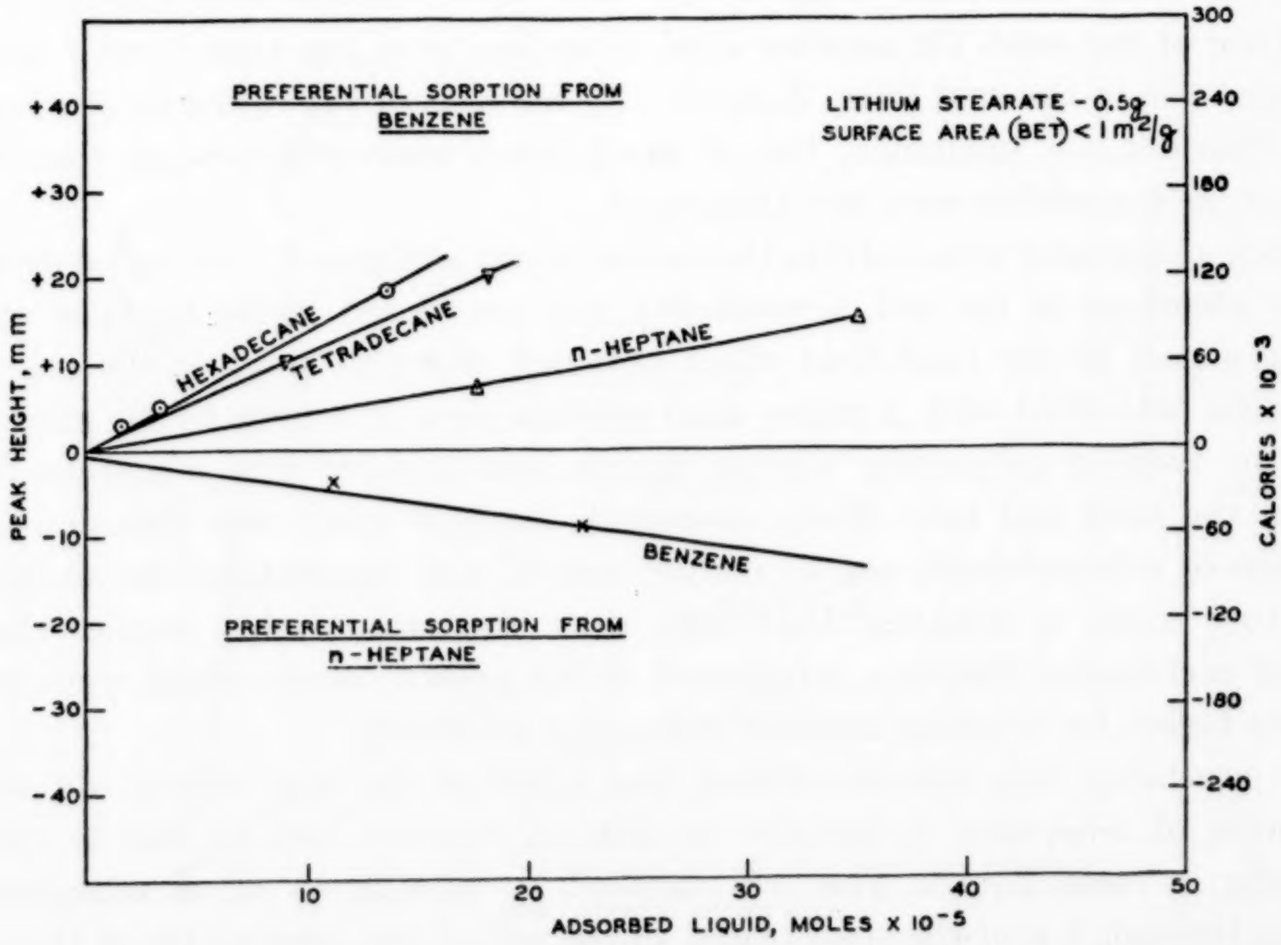

Fig. 4. Heats of preferential sorption on lithium stearate.

are plotted against the increasing amounts of liquids preferentially sorbed. The same relationships are shown in Figs. 5 and 6 for adsorptions on silica gel, and sorption on Zeocarb 225 (cation exchange resin) respectively.

## DISCUSSION AND CONCLUSIONS

The nature of the apparatus used in this work was such that the sorptions taking place were dynamic in character, *i.e.* the sorption that occurred was immediately followed by a desorption process, which usually, although not exclusively, produced a measurable heat effect opposite to that occurring in the adsorption process.

The heat effects produced by preferential adsorption have been studied under conditions in which the amount of the displacing liquid is insufficient to form a monomolecular layer on the available surface. All of the adsorbents used had surface areas in excess of 100 m²/g. For paraffinic molecules lying flat on such a surface, and occupying about 10 Å² per carbon atom, 100 m² of surface would accommodate $10^{21}$ such carbon atoms, equivalent to about 24 mg of hydrocarbon. For straight-chain organic molecules of molecular weight of 100 adsorbed end-on, and each occupying 16 Å², 100 m² of surface would accommodate about 100 mg. The quantities of displacing liquids used in this work were in the range 0.2 to 70 mg per gram of adsorbent and thus in general were insufficient to provide a monomolecular layer over these high surface area materials. On the other hand, one solid used (lithium stearate) had a surface area of less than 1 m²/g, which would be covered by less than 1 mg of adsorbate. Considerable heat effects obtained for this material indicate that mainly sorption processes take place, whereby the molecules of liquids enter into the crystalline lattice of the solid. On another solid of surface area less than 1 m²/g (a cation exchange resin in the acid form, Zeocarb 225) the heat of the exchange process with metal chlorides was examined, but at much lower concentrations, so that all the hydrogen ions available were not exchanged.

Since a substance injected into the carrier liquid is diluted by the liquid surrounding the adsorbent in the cell, a possibility was considered of the heats of solution forming a part of the total heat effect obtained. For this purpose the cell of the calorimeter was filled with a coarse sand (surface area $< 1$ m²/g by $N_2$ adsorption) having no sorptive properties. Various liquids were injected into *n*-heptane flowing through the sand and heat effects measured. No heat effect was obtained for the injections of *n*-hexadecane, and a positive heat of 0.03 cal obtained for an injection of $45 \cdot 10^{-5}$ moles of benzene. That heat effect was considerably smaller than the heats of preferential sorption determined in the present work, which were at least 10 times higher for a similar amount of benzene adsorbed.

A possibility was also considered that some of the heat effects obtained on adsorption of *n*-heptane or benzene on lithium stearate may be due to the soap dissolving in these liquids. This was checked by passing 50 ml of *n*-heptane and benzene through 1 g of the soap placed in the cell of the calorimeter at the rate of 0.4 ml/min, and filtering and evaporating the collected liquids. The residue was

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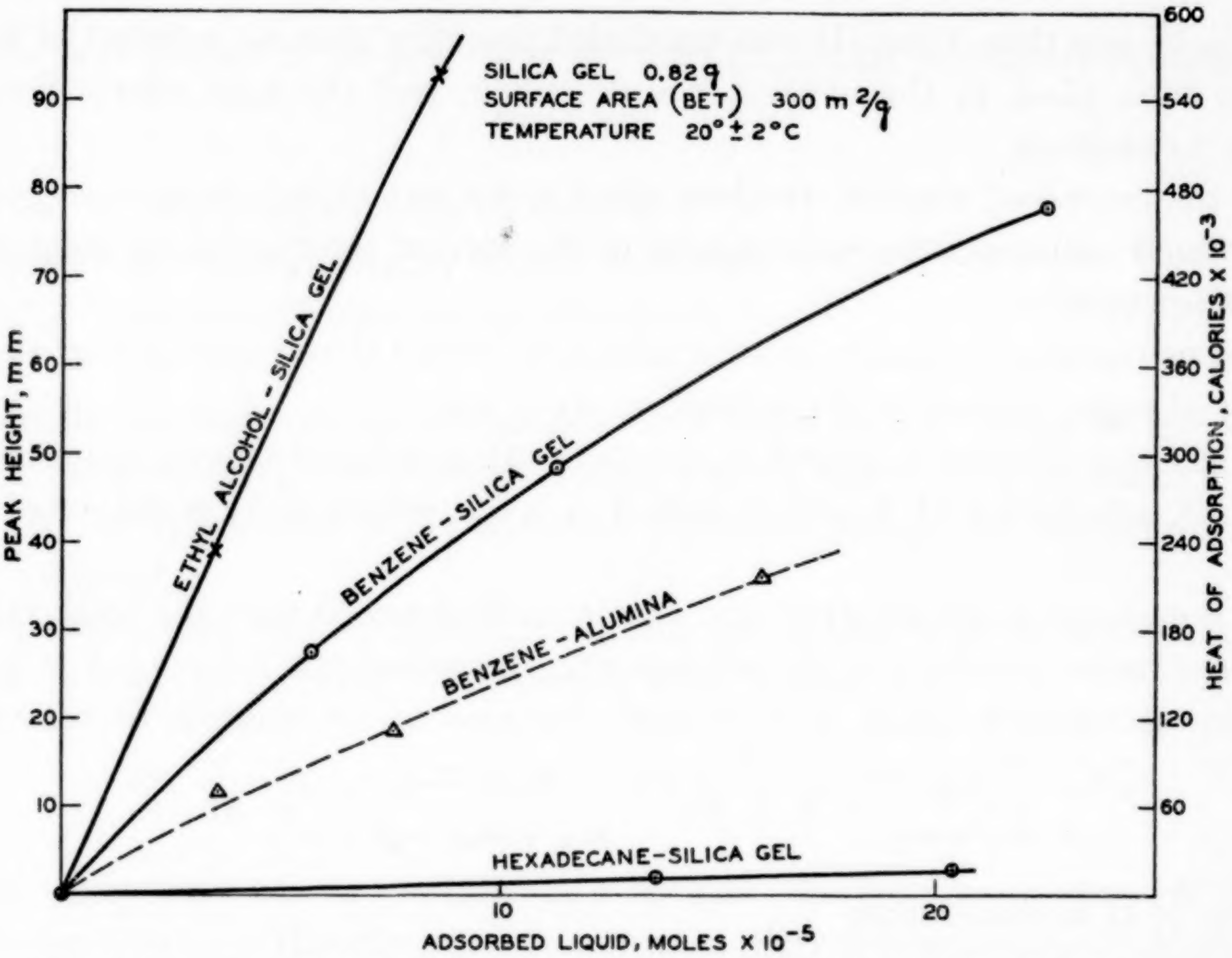

Fig. 5. Heats of preferential adsorption from *n*-heptane on silica gel and aluminium oxide.

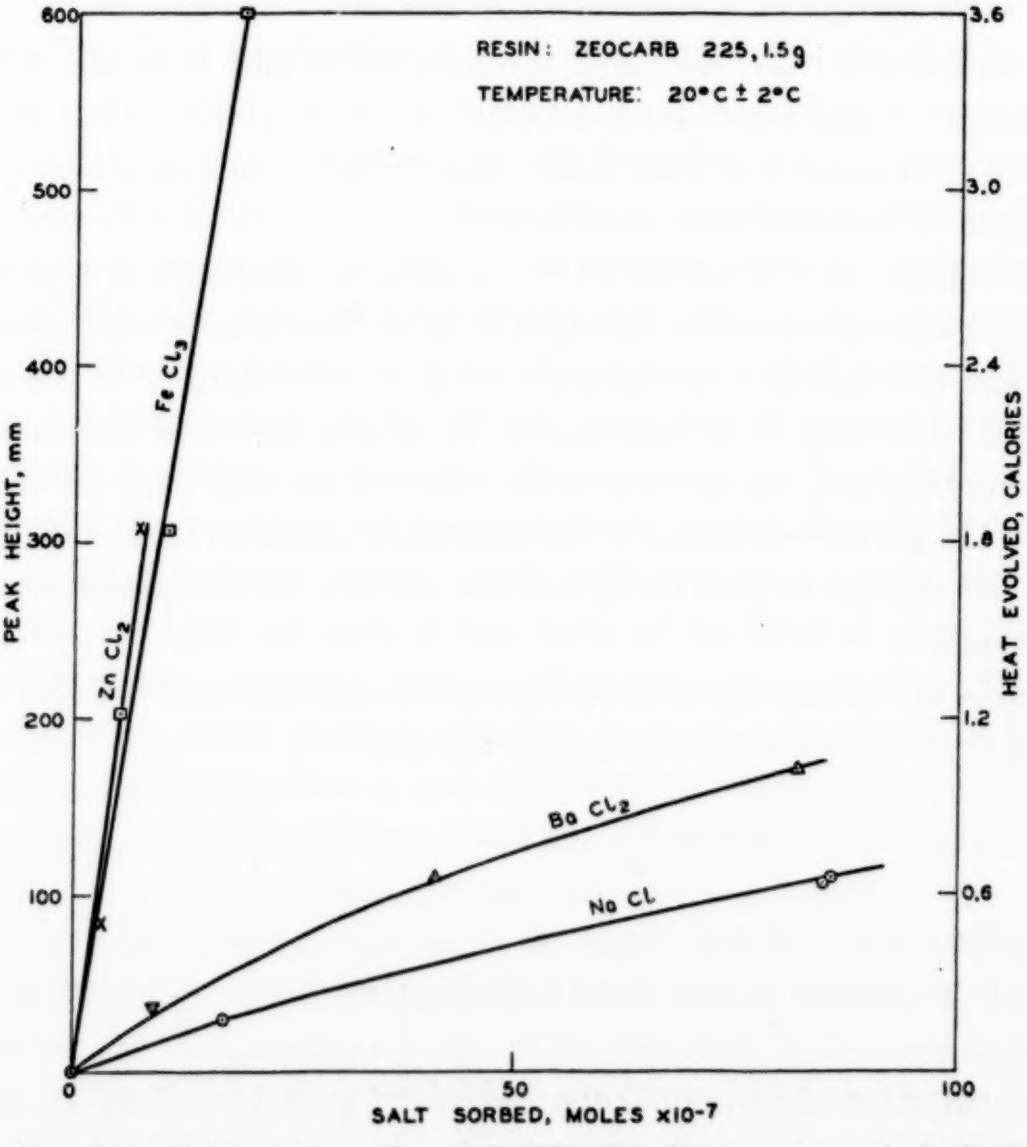

Fig. 6. Heats of preferential sorption of metal chlorides from water–ethyl alcohol solution on cation exchange resin in acid form.

found to be less than 1 mg. It was concluded therefore that no solution of lithium stearate took place in the experiments described, and the heat effects were due entirely to sorption.

In the process of sorption, the heat effect is due to a change in free energy of the solute which exchanges the environment of the solvent for that of the solid surface of the adsorbent[4, 5].

Let us consider a solution of substance A in solvent B and assume that the total number of moles present in the solution is $m_A + m_B$.

When this solution is placed in contact with a solid adsorbent saturated with solvent B, adsorption of A occurs only if it is accompanied by a decrease of free energy.

If $p$ moles of A are adsorbed and $r$ moles of B desorbed from the solid, the total number of moles remaining in the solution after the process will be $m_A - p + m_B + r$.

Thus the concentration of A in mole fractions in the solution decreases from

$$\frac{m_A}{m_A + m_B} = X \qquad \text{to} \qquad \frac{m_A - p}{m_A + m_B - p + r} = Xe$$

and that of B increases from

$$\frac{m_B}{m_A + m_B} = Y \qquad \text{to} \qquad \frac{m_B + r}{m_A + m_B - p + r} = Ye$$

where $X$, $Y$ and $Xe$, $Ye$ are the mole fractions of A and B in the solution before adsorption and at equilibrium after adsorption, respectively. Thus the adsorption and desorption processes continue until equilibrium concentrations of A and B, given by $Xe$ and $Ye$ respectively, are reached.

The total change in free energy of the system is composed of four main factors, *i.e.* those due to adsorption of A, desorption of B, decrease in concentration of A in the solution and increase in concentration of B in the solution. If the energy of the surface covered by solvent B is represented by $rS_B\gamma_B$ and that covered by solute A by $pS_A\gamma_A$, where $S_A$ and $S_B$ are the areas covered by one mole of the adsorbates, $\gamma_A$ and $\gamma_B$ are the surface tensions of the adsorbent covered with molecules A and B respectively, the change in free energy of the surface on the preferential adsorption is $pS_A\gamma_A - rS_B\gamma_B$.

The changes of free energy accompanying the decrease and increase in concentration of A and B in the solution can be represented by:

$$pRT \ln \frac{Xe}{X} \qquad \text{and} \qquad rRT \ln \frac{Ye}{Y}$$

respectively where $Xe < X$ and $Ye > Y$.

Thus the total change in free energy during the process of preferential adsorption is:

$$\Delta F = pS_A\gamma_A - rS_B\gamma_B + pRT \ln \frac{Xe}{X} + rRT \ln \frac{Ye}{Y} \qquad (1)$$

where $R$ is the gas constant and $T$ the absolute temperature.

The heat change in the process is given by the Gibbs–Helmholtz equation:

$$\Delta F = \Delta H - T\Delta S \qquad (2)$$

where $\Delta H$ = heat change during adsorption
$\Delta F$ = free energy change during adsorption
$\Delta S$ = entropy change during adsorption.

Usually the heat change is approximately equal to the change in free energy. In some cases however, the entropy change may be appreciable. In particular, this effect may be appreciable if the adsorbed molecules change the configuration they had in solution[5].

The heat $-\Delta H$ lost by the system during the process of preferential adsorption is given by eqn. (3) obtained by combining eqns. (1) and (2).

$$-\Delta H = -\left(pS_A\gamma_A - rS_B\gamma_B + pRT\ln\frac{Xe}{X} + rRT\ln\frac{Ye}{Y}\right) - T\Delta S \qquad (3)$$

For many adsorptions the difference between the first two factors of the free energy term is so large that the other factors are negligible in comparison. An example of this is the preferential adsorption of ethyl alcohol from solution in *n*-heptane on silica gel (Fig. 5). Thus, 0.270 cal of heat are evolved on adsorption of $4\cdot10^{-5}$ moles of the alcohol and the heat of dilution of the same amount of alcohol in *n*-heptane is only 0.020 cal.

According to theoretical predictions of DE BOER[7] it may be possible for sorption to occur with an endothermic heat effect, when the entropy change is sufficiently large and positive to allow $\Delta F$ in eqn. (2) to have a negative value in spite of the positive value of $\Delta H$. The preferential sorption of benzene from *n*-heptane on lithium stearate (Fig. 2a) may be a case in point. It may be added here that the heats of sorption of dry lithium stearate by *n*-heptane and benzene are both positive, but the heat for *n*-heptane is greater (3.00 cal/g) than that given by benzene (1.90 cal/g).

In liquid–solid chromatography the separation of constituents of a mixture may be influenced by all the factors on the right hand side of eqn. (3). The attempts to grade the eluting power of solvents on the basis of the heat of complete wetting of dry adsorbents[8] may be misleading if the pattern of adsorption of a substance, when preferential adsorption takes place, is different from the adsorption of the pure substance. The difference in question is one between the energy changes corresponding to terms $pS_A\gamma_A$ and $\Delta F$ given in eqn. (1). Thus, in general, it is considered that the determination of the heats of preferential sorption can predict the sequence of adsorption better than the determination of the heats of adsorption of dry adsorbents.

As can be seen in Fig. 2a and b, the positive heat effects are in most cases followed by negative heats. The latter heats are due to the desorption of material preferentially adsorbed when its concentration in the solution is below the equilibrium value *Xe*. Depending on this value, the negative heats are more or less marked. Thus, when ethyl alcohol is injected into a stream of benzene flowing through powdered lithium stearate, preferential sorption of ethyl alcohol takes place from benzene solution, the process continuing so long as the concentration of ethyl alcohol in the solution surrounding the adsorbent is above its equilibrium concentration $Xe$. When the concentration of ethyl alcohol in the solution becomes less than $Xe$, it is desorbed from lithium stearate, the process being accompanied by the absorption of heat.

If the value $Xe$ is very low, the desorption process may be very slow, and the absorption of heat occurring then is not indicated by the apparatus. Very small values of such negative heat effects characterize substances which are adsorbed very strongly from solution, *i.e.* substances for which the factor $pS_A\gamma_A$ is considerably greater than $rS_B\gamma_B$. An example of this type of adsorption is furnished by preferential adsorption of ethyl alcohol on carbon black from *n*-heptane solution (Fig. 2b).

Most of the curves given in Figs. 3 to 6 show clearly that the heats of preferential adsorption and sorption decrease as the amount of substances adsorbed increases. This effect is very similar to that exhibited by the heats of adsorption of liquid and gaseous substances on solid surfaces[9].

It is concluded that the solid surfaces studied in this work are not uniform in character and consequently the heat evolved when the first molecules of a substance are adsorbed is higher (when expressed as a differential heat of adsorption) than that produced when the surface is partly saturated with the absorbate.

It may be expected therefore that when a small amount of a substance is absorbed on the same weight of identical adsorbents possessing different surface areas, the solid with the greatest surface area would give the highest heat of preferential sorption. This has been in fact found for silica gel where the adsorption of 20 $\mu$l of benzene from solution in *n*-heptane on the gel possessing the area of 700 $m^2/g$ gives a heat effect of 0.570 cal, whereas the same amount of benzene adsorbed on the gel with a surface area of 300 $m^2/g$ gives 0.450 cal.

The heat effects shown in Fig. 6 correspond to the process of sorption involving strong ionic forces. When the metal chlorides come into contact with the resin in its acid form, a reaction sets in, whereby a metal salt of the resin is produced together with a corresponding amount of hydrochloric acid. The heat effect is mainly the result of the differences in the heat produced on the formation of the resin–metal complex and that absorbed in the decomposition of the chloride with the subsequent formation of hydrochloric acid and its solution in the water–alcohol mixture used as the carrier liquid.

It is interesting to note that the differential heats of preferential sorption that can be obtained from the curves shown in Fig. 6 decrease continuously, which is similar to the trend exhibited by the preferential adsorption.

It is inferred from the results shown in Figs. 3 and 5, for processes in which the entropy change is negligible, that if mixtures of the substances studied (*e.g.* *n*-heptane–benzene, benzene–ethyl alcohol, etc.) were passed through columns filled with the respective adsorbents, the separations achieved would be predicted by the order of the heats of preferential sorption, the substance with a lower heat being the first to emerge from the column.

## ACKNOWLEDGEMENTS

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## SUMMARY

The construction and applications of an inexpensive calorimeter suitable for the determination of heats of preferential sorption from liquid mixtures on solid surfaces is described. The calorimeter is very sensitive and is capable of detecting the heat effects as low as 0.001 cal/g.

The application of the calorimeter is illustrated by determinations of the heats of sorption for a number of liquids dissolved in *n*-heptane and benzene on solid adsorbents such as carbon black, silica gel, alumina, lithium stearate and Zeocarb 225 (a cation exchange resin).

The significance of the results is discussed with special reference to the theory of solid–liquid chromatography.

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# RELATIVE DETECTOR RESPONSE IN GAS CHROMATOGRAPHY

G. R. JAMIESON
*Chemistry Department, The Paisley Technical College, Paisley (Great Britain)*

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In recent years a considerable amount of work has been carried out using gas chromatography for quantitative analysis of mixtures of organic compounds, and, in many cases, the technique has replaced infra-red and mass spectrometric determinations. A comparison of various types of differential detectors used in gas chromatography has been made by McWILLIAM[1]. He discusses the suitability of the different detectors for quantitative analysis and he also discusses the desirability of there being a direct relationship between the signal output of a detector and some molecular parameter; he concludes that a detector output equal for all compounds on a weight basis would be the most convenient.

Up to the present time the differential detector which has been most used has been the thermal conductivity cell and quantitative analysis with this type of detector has been discussed by KEULEMANS *et al.*[2] and for the most accurate analysis it is agreed that calibration of the detector is necessary. However, calibration is not always possible as the pure components of a mixture may not be readily available. Various corrections have been applied to the recorded peak area percentages to obtain better agreement with the actual weight percentages, *e.g.* BROWNING AND WATTS[3] employ a correction for the thermal conductivity of the component, and EASTMAN[4] employs a correction involving the square root of the molecular weight of the component. These workers apply the corrections to results obtained with helium as the carrier gas. The usefulness of these corrections when nitrogen is the carrier gas has been discussed by the author[5].

With helium as the carrier gas, the detector responses for a large number of compounds of different types have been reported[6,7], and it was found convenient to report these responses relative to the detector response given by a comparison compound, benzene. From the results obtained it was shown that there is a direct relationship between relative detector response and molecular weight in a given homologous series and the effect of chain branching within a series was also shown.

The purpose of the present investigation was to examine the relative response of thermal conductivity detectors to compounds of various types when nitrogen was the carrier gas. As the temperature range investigated was greater than that used by MESSNER *et al.*[7] it was not found possible to relate all the responses to that of one comparison compound. Three comparison compounds were used; benzene for temperatures up to 95°, *p*-cymene at a temperature of 160°, and methyl palmitate at a temperature of 210°.

The behaviour of thermal conductivity detectors when nitrogen is the carrier gas has been discussed by BOHEMEN AND PURNELL[8] with particular reference to partial and complete reversal of peaks. Such reversal of peaks has been found by many workers in this field[2,9-11] and various explanations for their occurrence have been advanced[2,9,10]. BOHEMEN AND PURNELL showed that peak reversal is both temperature and flow-rate dependent. Therefore, for satisfactory quantitative analyses based on peak areas, the operating conditions must be chosen such that peak reversal does not occur.

As more data on relative detector response becomes available and if the conditions of measuring the relative response become standardised, then relative response would be an important adjunct to retention volume for the identification of an unknown compound by the gas chromatographic method.

## EXPERIMENTAL

### *Apparatus*

Gas–liquid partition chromatography was carried out using a Griffin and George Mk IIA apparatus equipped with thermal conductivity detectors. Nitrogen was used as the carrier gas at a flow rate of approximately 33 ml/min.

The bridge current was 100 mA except for the determinations of the methyl esters of long-chain aliphatic acids when the bridge current was increased to 130 mA.

The liquid phases used were di-iso-octyl sebacate and silicone E301 supported on Celite 545 (60–80 mesh) and 6 ft. glass columns were used. For the determinations of the methyl esters of long-chain aliphatic acids, Apiezon L supported on Celite 545 (60–80 mesh) was used and good separations were obtained using a 3 ft. glass column.

### *Materials*

Liquids were dried using standard methods and finally purified by fractional distillation. Solids were recrystallised to constant melting point. Small amounts of the following compounds were purified by preparative scale gas chromatography: α-pinene, β-pinene, camphene, limonene and myrcene.

### *Procedure*

Each compound to be investigated was mixed with a known amount of the comparison compound and the resulting blends were run a minimum of three times. A minimum of two blends was prepared for each compound. The volume of sample injected into the chromatographic column was not known accurately but was between 0.01 and 0.02 ml.

The areas under the peaks were found by multiplying the height of the peak by the width at half peak height. The comparison compound was arbitrarily assigned a signal response of 100 units per mole and the response of the other compounds were calculated relative to the comparison compound.

## RESULTS AND DISCUSSION

The relative responses for a number of compounds of different types are shown in Table I.

An examination of the response data relative to benzene shows that, in a homologous series, there is an increase in relative response with molecular weight. The increase in relative response with molecular weight is similar for different homologous

TABLE I

| *Compound* | *Response per mole relative to benzene* | *Compound* | *Response per mole relative to p-cymene* |
|---|---|---|---|
| *Aromatic hydrocarbons* | | *Hydrocarbons* | |
| Benzene | 100 | *p*-Cymene | 100 |
| Toluene | 121 | Camphene | 79 |
| Ethylbenzene | 144 | α-Pinene | 73 |
| *n*-Propylbenzene | 162 | β-Pinene | 72 |
| *p*-Cymene | 180 | Limonene | 83 |
| *Methyl ketones* | | Myrcene | 82 |
| Acetone | 65 | *Alcohols* | |
| Butan-2-one | 80 | Phenol | 48 |
| Pentan-2-one | 97 | Cyclohexanol | 51 |
| 3-Methylbutan-2-one | 95 | α-Terpineol | 82 |
| Mesityl oxide | 114 | Menthol | 76 |
| Hexan-2-one | 118 | Geraniol | 115 |
| 4-Methylpentan-2-one | 116 | Linalool | 113 |
| 3,3-Dimethylbutan-2-one | 110 | Citronellol | 118 |
| Heptan-2-one | 136 | *Ketones* | |
| Methyl heptenone | 144 | Acetophenone | 81 |
| Acetophenone | 145* | Isophorone | 75 |
| *Alcohols* | | Menthone | 76 |
| Methanol | 19 | Camphor | 75 |
| Ethanol | 34 | ψ-Ionone | 138 |
| Propan-1-ol | 57 | β-Ionone | 128 |
| Propan-2-ol | 49 | Geranyl acetate | 168 |
| Allyl alcohol | 50 | | |
| Butan-1-ol | 87 | | |
| 2-Methylpropan-1-ol | 80 | | *Response per mole relative to methyl palmitate* |
| Butan-2-ol | 72 | | |
| 1,1-Dimethylethanol | 61 | | |
| Pentan-1-ol | 116 | *Methyl esters* | |
| Cyclohexanol | 92* | Methyl decoate | 69 |
| Phenol | 87* | Methyl laurate | 78 |
| *Esters* | | Methyl myristate | 90 |
| Methyl acetate | 98 | Methyl palmitate | 100 |
| Ethyl acetate | 119 | Methyl stearate | 109 |
| *n*-Propyl acetate | 137 | Methyl arachidate | 120 |
| Isopropyl acetate | 133 | | |
| Ethyl propionate | 133 | | |
| *n*-Butyl acetate | 158 | | |
| Isobutyl acetate | 157 | | |
| *sec.*-Butyl acetate | 146 | | |
| *tert.*-Butyl acetate | 139 | | |
| *n*-Amyl acetate | 178 | | |

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* Calculated from the value relative to *p*-cymene.

series being approximately 20 units per $CH_2$ group. Also, for isomeric compounds, there is a decrease in relative response with increase in chain branching, and this decrease is more marked with alcohols than with either the alkyl acetates or methyl ketones.

The relationship between relative response and the square root of the molecular weight shown by EASTMAN[4] cannot be generally applied when nitrogen is the carrier gas. The graph of relative response *vs.* square root of molecular weight is shown in Fig. 1 for aliphatic straight-chain alcohols and even in this series the first members do not fall on the straight line.

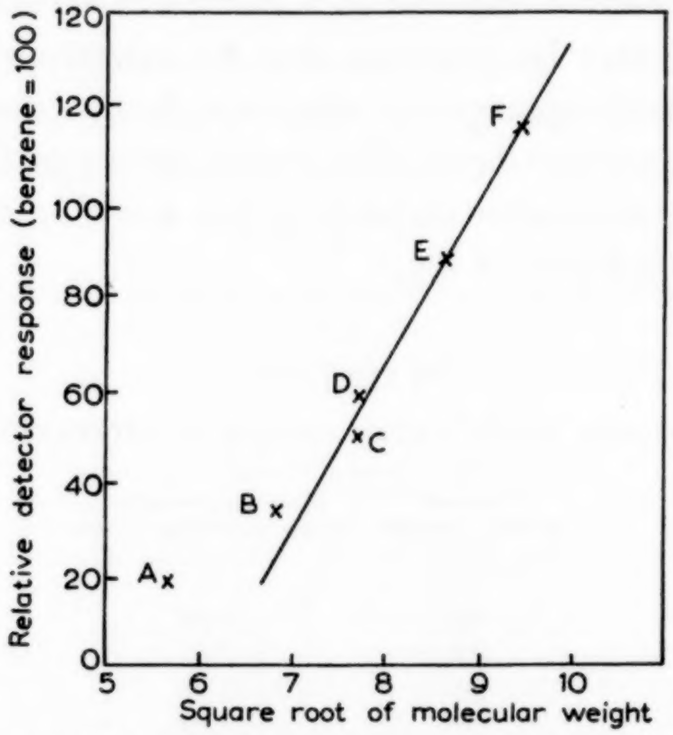

Fig. 1. Relation between relative detector response to aliphatic alcohols and square root of molecular weight. A: methanol; B: ethanol; C: allyl alcohol; D: propan-1-ol; E: butan-1-ol; F: pentan-1-ol.

An examination of the response data relative to *p*-cymene shows that compounds of similar type have similar responses and this data has been used for a semi-quantitative analysis of essential oils when the pure components were not available for calibration purposes.

The relative responses of the methyl esters of the long-chain aliphatic acids increase regularly with molecular weight. It has been reported previously[12, 13] that

TABLE II

QUANTITATIVE DATA ON METHYL ESTERS OF LONG-CHAIN ALIPHATIC ACIDS

Column: 3 ft. Apiezon L on Celite; Temperature: 210°; Flow rate: 33 ml/min, nitrogen

| *Ester* | *Weight %* | | *Area %* | |
|---|---|---|---|---|
| Methyl decoate | 30.6 | — | 30.4 | — |
| Methyl laurate | 10.7 | — | 10.5 | — |
| Methyl myristate | 25.1 | 19.0 | 25.5 | 18.9 |
| Methyl palmitate | 33.6 | 23.8 | 33.6 | 24.1 |
| Methyl stearate | — | 24.5 | — | 24.1 |
| Methyl arachidate | — | 32.7 | — | 32.9 |

these compounds can be analysed quantitatively without calibrating the detector and this is confirmed in the present work (Table II).

### *Relative response and flow rate*

BOHEMEN AND PURNELL[8] have discussed the flow-rate dependence of the temperature at which peak inversion occurs when nitrogen is the carrier gas and so it would be wrong to assume that relative response to a particular substance is independent of flow rate of nitrogen. It may be, however, that the relative response to a particular compound does not change appreciably when the flow rate of carrier gas is varied between comparatively narrow limits. The results shown in Table III indicate that there is little change in the relative response to *tert.*-butyl acetate when the flow rate of nitrogen is varied by a factor of 2.5.

TABLE III

RELATIVE RESPONSE TO *tert.*-BUTYL ACETATE AT DIFFERENT FLOW RATES

| *Flow rate ml/min, nitrogen* | *Response per mole relative to benzene = 100* |
|---|---|
| 17 | 140 |
| 23 | 142 |
| 35 | 139 |
| 42 | 139 |

### *Relative response and temperature*

MESSNER *et al.*[7] report that, when helium is the carrier gas, relative response values are independent of temperature over a range from 30° to 160°. Using nitrogen as the carrier gas it is unlikely that such a temperature independence would be found since it has been found[8] that the phenomenon of peak inversion is temperature dependent.

TABLE IV

RELATIVE RESPONSE AT DIFFERENT DETECTOR TEMPERATURES

| *Temperature °C* | *Response per mole relative to benzene = 100* | |
|---|---|---|
| | *Cyclopentanone* | *Cyclohexanone* |
| 81 | 109 | 114 |
| 98 | 108 | 105 |
| 116 | 106 | 99 |
| 130 | 105 | 92 |
| | *Response per mole relative to p-cymene = 100*<br>*n-Butyl acetate* | |
| 105 | 86 | |
| 120 | 87 | |
| 138 | 86 | |
| 152 | 86 | |

In the present work no indication of peak inversion was observed with any of the compounds used at the operation temperatures of the detector. Even under these circumstances, however, a variation in relative response with detector temperature was observed with cyclopentanone and cyclohexanone but not with *n*-butyl acetate (Table IV).

### *Reproducibility*

It is necessary to examine the relative responses given by similar detectors since MESSNER *et al.*[7] conclude that their data should be applicable to all gas chromatographs using thermal conductivity detectors and helium as the carrier gas. A comparison of

TABLE V

COMPARISON OF RELATIVE RESPONSE TO DIFFERENT DETECTORS WITH HELIUM AS THE CARRIER GAS

| *Compound* | *Response per mole relative to benzene = 100* | | |
|---|---|---|---|
| | *Detector* | | |
| | *1** | *2*** | *3**** |
| Benzene | 100 | 100 | 100 |
| Toluene | 116 | 116 | — |
| *o*-Xylene | 130 | 130 | — |
| *p*-Xylene | 131 | 133 | — |
| *n*-Heptane | 143 | 135 | — |
| *n*-Octane | 160 | 156 | — |
| Cyclopentane | 97 | 91 | — |
| Cyclohexane | 114 | 116 | — |
| Methylcyclopentane | 115 | 105 | — |
| Methylcyclohexane | 120 | 120 | — |
| Water | 21 | — | 21 |
| Butan-1-ol | 95 | — | 95 |
| *n*-Butyl acetate | 135 | — | 127 |
| Di-*n*-butyl ether | 160 | — | 155 |

* Results of MESSNER *et al.*[7]
** Results of NUNEZ *et al.*[14] recalculated on a mole basis.
*** Results of HASKIN *et al.*[15] recalculated on a mole basis.

TABLE VI

COMPARISON OF RELATIVE RESPONSE TO DIFFERENT DETECTORS WITH NITROGEN AS THE CARRIER GAS

| *Compound* | *Response per mole relative to benzene = 100* | | |
|---|---|---|---|
| | *Detector* | | |
| | *1** | *2** | *3*** |
| Benzene | 100 | 100 | 100 |
| Toluene | 120 | 121 | 120 |
| Chloroform | 179 | — | 182 |
| Carbon tetrachloride | 253 | — | 255 |
| Acetone | 65 | 65 | — |
| Ethanol | 34 | 35 | — |
| Propan-1-ol | 57 | 56 | — |
| Propan-2-ol | 49 | 49 | — |

* Present work.
** Results of GRANT[16] recalculated on a mole basis.

their results and those of other investigators using similar instruments is shown in Table V. The results shown in Table VI indicate that there is an agreement between the detectors used in the present investigation and a similar detector. However there is, as yet, not enough published work on relative responses to show to what extent the reproducibility exists.

## CONCLUSION

A correlation is shown to exist between relative detector response and molecular weight for a given homologous series. With nitrogen as the carrier gas it is important that the detector temperature and the flow rate of nitrogen should be chosen such that no peak inversion takes place. Further investigation is required on a wider range of compounds and also on the relative detector response using various types of thermal conductivity detector. As this data becomes available then relative detector response could be used in conjunction with retention volume data for identification pusposes.

## ACKNOWLEDGEMENTS

Thanks are due to Miss M. McKellar for carrying out many of the determinations, and Mr. W. Hudson, Roche Products Ltd. (Dalry), for the gift of a pure sample of α-terpineol and for samples of the ionones.

## SUMMARY

The response of thermal conductivity detectors in gas chromatography to compounds of different types is shown relative to comparison compounds and the effect of detector temperature and carrier gas flow rate is discussed. Results are compared when helium and when nitrogen is the carrier gas.

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# ANALYSIS AND COMPOSITION OF OIL OF LEMON BY GAS–LIQUID CHROMATOGRAPHY*

RICHARD A. BERNHARD

*Department of Food Science and Technology, University of California, Davis, Calif. (U.S.A.)*

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Prior to the advent of gas–liquid chromatography (GLC), the analysis of essential oils was a laborious and all too frequently unrewarding research problem. Even a cursory examination of PCORE'S[1] analyses of orange and lemon oils will illustrate these points. Early attempts to utilize the technique of gas–liquid chromatography gave no notable improvements in this area. BERNHARD[2] was able to obtain only five distinct peaks on examination of lemon oil employing primitive gas–liquid chromatographic apparatus. It appears now that the detectors employed in this early study were insufficiently sensitive to determine components present in minor amounts. Additionally, no thorough search was conducted to determine the best stationary liquid phase for carrying out these separations. After this publication[2], work was initiated to determine what compound or compounds were best suited to serve as a stationary liquid phase for the separation and identification by GLC of the components present in citrus oils. The results of that study[3] indicated that there are three liquids which can serve as suitable stationary liquid phases; they are LAC-2-R446 (the adipate polyester of diethylene glycol partially cross-linked with pentaerythritol), LAC-4-R777 (the succinate polyester of diethylene glycol), and Craig polyester adipate. Although LIPSKY AND LANDOWNE[4,5] had previously reported on the use of LAC-2-R446 and LAC-4-R777 for the separation of unsaturated fatty acid esters, no one has reported their use to separate the components of essential oils[3]. Use of these three stationary phases affords a rather extensive separation of the components present in oil of lemon.

## EXPERIMENTAL

The apparatus employed to separate the constituents of the lemon oils was an Aerograph model A-90-C (manufactured by Wilkins Instrument and Research, Inc., Walnut Creek, Calif.). Columns were constructed of stainless steel tubing, ¼ inch O.D. and 10 feet in length. The support material used throughout this study was Sil-O-Cel C-22 diatomaceous earth firebrick (30 to 60 mesh). Fractions of this were sieved to size and further graded by sedimentation in water, after which they were dried intensively. The liquid phase was introduced by deposition from benzene solution,

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* Supported in part by a grant from the Sunkist Growers, Inc., Los Angeles, Calif.

the packing then being heated in a stream of helium to a temperature 20–30° higher than any used in the subsequent experiments.

The column was contained in an air thermostat; the carrier gas flow was controlled by a precision reducing valve and measured by a soap bubble flow meter situated at the column outlet. Temperature and flow control were sufficiently good to make possible the use of a four-channel, hot wire katharometer that proved virtually noiseless when connected with a 1 mV recording potentiometer. Sample injection was made by a microsyringe, and the column preheater was maintained at 50° above the temperature at which the column was operated.

Separations were carried out with the aid of two stationary liquid phases: LAC-2-R446[4] and LAC-4-R777[5]. The materials were applied to the solid support in the amount of 25 % w/w.

The parameters of operation were as follows: column temperature 150°; helium flow rate 90 ml/min; sample volume 5 to 20 $\mu$l; recorder chart speed 30 in./h. The column outlet was maintained at atmospheric pressure.

The lemon oil samples examined were cold-pressed California oils obtained from the Sunkist Growers, Inc., of Los Angeles, California. They were from last year's crop of fruit (April–May) and were so-called raw oil samples. No antioxidants were added. A typical oil had the following physical properties: specific gravity $25°/25° = 0.852$; citral content = 3.40 % (actually measured as total aldehyde content); $\alpha_D^{25°}$ + 55.33°; $n_D^{20°}$ 1.4749.

## RESULTS AND DISCUSSION

When cold-pressed California lemon oil was examined by means of GLC employing a stationary liquid phase of LAC-2-R446, thirty-two peaks were evident on the chromatogram (Fig. 1); twenty-two of these peaks have been numbered for purposes

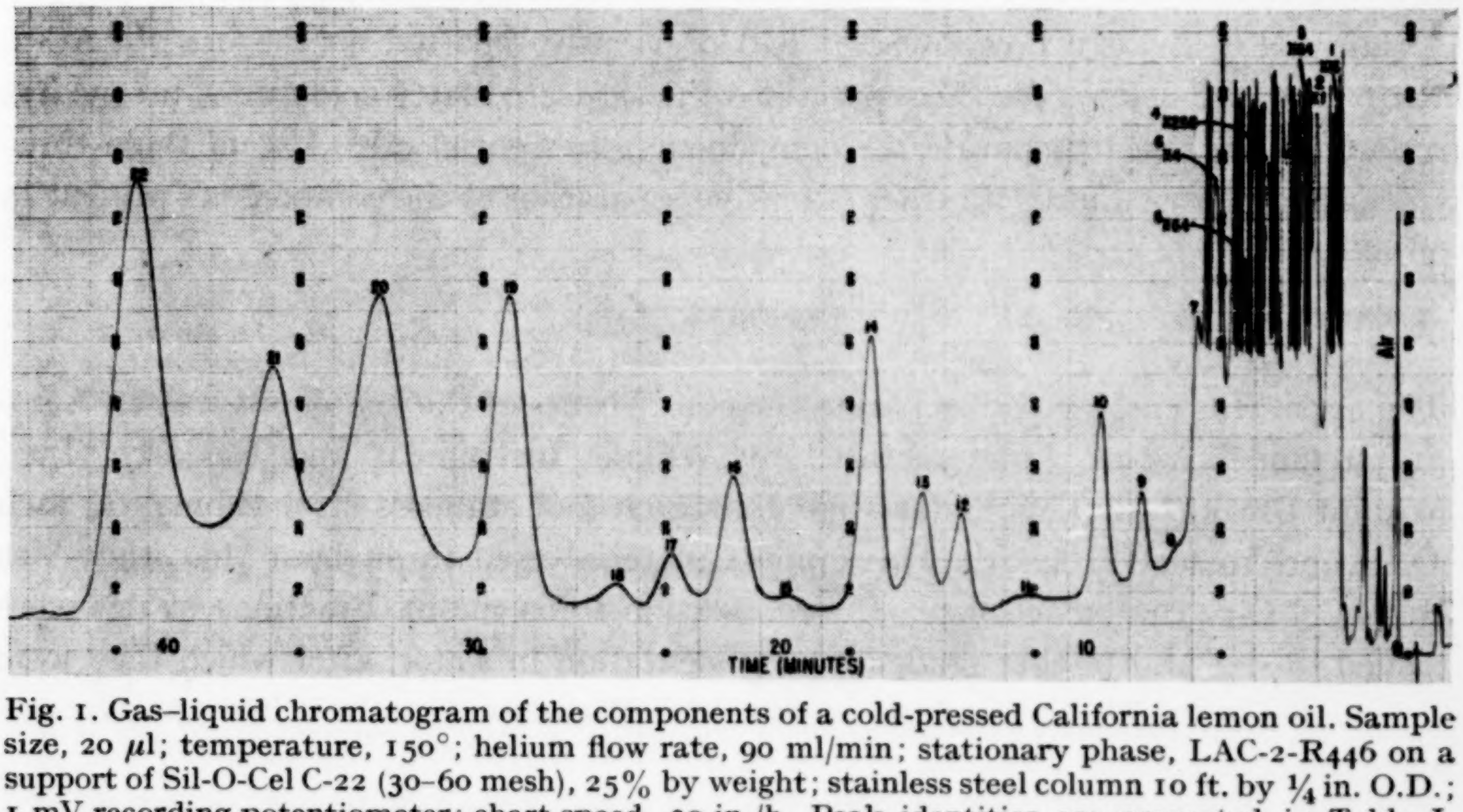

Fig. 1. Gas–liquid chromatogram of the components of a cold-pressed California lemon oil. Sample size, 20 $\mu$l; temperature, 150°; helium flow rate, 90 ml/min; stationary phase, LAC-2-R446 on a support of Sil-O-Cel C-22 (30–60 mesh), 25% by weight; stainless steel column 10 ft. by ¼ in. O.D.; 1 mV recording potentiometer; chart speed, 30 in./h. Peak identities are presented in Table I.

of identification. It may be seen that there are ten small peaks near the origin of the chromatogram (between the air peak and peak 1) which are not numbered. These peaks differ in both position, that is, retention distance, and amount, from sample to sample and have not as yet presented any consistent pattern. The peaks numbered from 1 through 22 differ generally only in amount from sample to sample. Thus the principal investigations and identifications were concerned with these peaks or components (work is currently in progress on the identification of the first ten or so peaks in various oil samples). The assignment of various peak identities was made by determination of the corrected retention volumes ($V_R^\circ$)[6] of known compounds and comparison with those for the unknown peaks. In this manner, a tentative identification of a large number of the components present was achieved. Confirmation of these results was obtained by an enrichment procedure in which known compounds were added, one at a time, to fresh portions of the lemon oil and re-examined by GLC. Data are presented in the form of relative retention volumes ($V_R^\circ/V_R^\circ$)[6] (Table I). It should be noted that it is experimentally impossible to distinguish between two compounds

TABLE I

RELATIVE RETENTION VOLUMES OF THE COMPONENTS OF COLD-PRESSED CALIFORNIA LEMON OIL

Stationary phase LAC-2-R446; temperature 150°; helium flow rate 90 ml/min. *n*-Decanal = 1.00.

| *Peak* | $V_R^\circ/V_R^\circ$ | | *Compound* |
|---|---|---|---|
| | *Unknown* | *Known* | |
| 1 | 0.144 | 0.148 | α-pinene |
| 2 | 0.189 | — | — |
| 3 | 0.217 | 0.227 | (β-pinene; myrcene) |
| 4 | 0.299 | 0.298 | *d*-limonene |
| | | 0.302 | *n*-heptanal |
| 5 | 0.352 | 0.351 | 1,8-cineole |
| | | 0.358 | γ-terpinene |
| 6 | 0.420 | — | — |
| 7 | 0.454 | 0.444 | (*n*-hexanol) |
| | | 0.455 | *n*-octanal |
| 8 | 0.501 | — | — |
| 9 | 0.583 | 0.571 | (methyl heptenone) |
| 10 | 0.679 | 0.676 | *n*-nonanal |
| 11 | 0.842 | 0.840 | octyl acetate |
| 12 | 1.00 | 1.00 | *n*-decanal |
| 13 | 1.08 | 1.06 | linalool |
| 14 | 1.21 | 1.18 | citronellyl acetate |
| 15 | 1.41 | 1.43 | citronellal |
| | | 1.47 | (*n*-undecanal) |
| 16 | 1.53 | 1.54 | tetrahydrogeraniol |
| 17 | 1.67 | — | — |
| 18 | 1.81 | 1.83 | decyl acetate |
| 19 | 2.06 | — | — |
| 20 | 2.36 | 2.30 | (α-terpineol) |
| | | 2.38 | citronellol and/or neral |
| 21 | 2.61 | 2.61 | geranyl acetate |
| 22 | 2.93 | 2.90 | citral |

whose relative retention volumes differ by 7 % or less since they will appear on the chromatogram as a single, united peak; differences of 10 to 15 % in relative retention volumes will show peaks that are united, *e.g.*, shoulders, or doublets; and differences of 20 % or more are necessary for complete separation of zones or peaks[7,8].

In order to be well within the bounds of experimental error, an arbitrary limit of agreement not to exceed 2 % was established. Compounds with values for corrected relative retention volumes not agreeing to within 2 % of each other are enclosed in parentheses. In Table I, those peaks that differ by more than the 2 % limit are: peak (3) α-pinene, myrcene (4.4 % difference); peak (7) *n*-hexanol (2.3 % difference); peak (9) methyl heptenone (2.1 % difference); peak (15) citronellal (2.8 % difference); *n*-undecanal (4.1 % difference); and peak (20) α-terpineol (2.5 % difference). These differences are well within the 7 % limit found by JAMES[7] and BERNHARD[8].

TABLE II

RELATIVE RETENTION VOLUMES OF THE COMPONENTS OF COLD-PRESSED CALIFORNIA LEMON OIL

Stationary phase LAC-4-R777; temperature 150°; helium flow rate 90 ml/min. *n*-Decanal = 1.00.

| *Peak* | $V_R°/V_R°$ | | *Compound* |
|---|---|---|---|
| | *Unknown* | *Known* | |
| 1 | 0.144 | 0.136 | (α-pinene) |
| 2 | 0.187 | — | — |
| 3 | 0.219 | 0.217 | β-pinene; myrcene |
| 4 | 0.299 | 0.294 | *d*-limonene |
| 5 | 0.358 | 0.343 | (γ-terpinene) |
| | | 0.363 | 1,8-cineole |
| 6 | 0.417 | — | — |
| 7 | 0.492 | 0.495 | *n*-octanal |
| 8 | 0.561 | — | — |
| 9 | 0.701 | 0.701 | *n*-nonanal |
| 10 | 0.824 | — | — |
| 11 | 1.00 | 1.00 | *n*-decanal |
| 12 | 1.07 | 1.07 | citronellal |
| 13 | 1.14 | 1.15 | linalool |
| | | 1.18 | linalyl acetate |
| 14 | 1.26 | — | — |
| 15 | 1.40 | 1.41 | *n*-undecanal |
| 16 | 1.85 | 1.80 | citronellyl acetate |
| 17 | 2.09 | 2.01 | (*n*-dodecanal) |
| 18 | 2.48 | 2.41 | (α-terpineol and/or geraniol) |
| | | 2.46 | citronellol |
| 19 | 2.73 | 2.74 | geranyl acetate |
| 20 | 3.21 | 3.22 | citral |
| 21 | 3.59 | 3.68 | (*d*-carvone) |

As a further check on identity, relative retention volumes were evaluated on a second stationary liquid phase, LAC-4-R777 (Table II). The data supplied by the use of another liquid phase lend credence to the tentative identification of the compounds present in the oil. Employing a liquid phase of LAC-4-R777, lemon oil showed twenty-one major peaks on the chromatogram. Peak identities were assigned on the basis of agreement of relative retention volumes for the unknown peaks with those values for known compounds. Those peaks that differ by more than the 2 % limit of agreement are: peak (1) α-pinene (5.8 % difference); peak (5) γ-terpinene (4.4 % difference); peak (17) *n*-dodecanal (4.0 % difference); peak (18) α-terpineol and/or geraniol (2.9 % difference); and peak (21) *d*-carvone (2.4 % difference). Once again these differences are well within the 7 % limit[7,8].

The chemistry of cold-pressed California lemon oils was investigated by POORE[1] who identified the following compounds: α-pinene, β-pinene, *d*-limonene, γ-terpinene, citral, *n*-octanal, *n*-nonanal, geraniol, linalool, a tertiary alcohol (?), a crystalline aldehyde, acetic, caprylic, caproic acids, bisabolene, and cadinene. POORE[1] could not find any methyl heptenone in California lemon oils, although this compound was reported to occur in Italian lemon oils by workers at Schimmel & Co.[9]. The results of the investigation described herein agree well with most of the findings of POORE. In addition to those compounds reported by POORE, a number of esters were detected, and the presence of methyl heptenone appears likely. It may well be that POORE's methods of isolation, *i.e.*, steam distillation, hydrolysis, etc., did not permit the recovery of intact ester components present in the oils.

A proximate composition of a typical cold-pressed California lemon oil was determined by integrating the areas under the appropriate peaks (Fig. 1) and is presented in Table III. It should be noted that these values do not represent either the

TABLE III

PROXIMATE COMPOSITION OF A TYPICAL COLD-PRESSED CALIFORNIA LEMON OIL

| *Peaks* | *Compound** | *Per cent composition*** |
|---|---|---|
| First 10 unnumbered peaks | — | 0.06 |
| 1 | α-pinene | 2.65 |
| 2 | — | 0.19 |
| 3 | β-pinene; myrcene | 12.69 |
| 4 | *d*-limonene; *n*-heptanal | 72.35 |
| 5 | γ-terpinene; cineole | 8.50 |
| 6 | — | 0.74 |
| 7 | *n*-octanal; (*n*-hexanol) | 0.15 |
| 8 | — | 0.09 |
| 9 | (methyl heptenone) | 0.06 |
| 10 | *n*-nonanal | 0.09 |
| 11 | octyl acetate | 0.04 |
| 12 | *n*-decanal | 0.06 |
| 13 | linalool | 0.08 |
| 14 | citronellyl acetate | 0.17 |
| 15 | citronellal; (*n*-undecanal) | 0.03 |
| 16 | tetrahydrogeraniol | 0.11 |
| 17 | — | 0.06 |
| 18 | decyl acetate | 0.05 |
| 19 | — | 0.32 |
| 20 | citronellol; neral | 0.51 |
| 21 | geranyl acetate | 0.40 |
| 22 | citral | 0.61 |
| Total | | 100.01 |

* Peak assignments based upon data from Table I.
** Values based upon integration of areas under appropriate peaks (Fig. 1).

precise weight per cent or mole per cent of the components in question, but some intermediate value that differs from either of these by a small but significant factor (usually from 1 to 5 %)[10]. The actual katharometer response may vary considerably with the nature of the components, and thus the output signal may not be linear with concentration in a highly varied, multicomponent system[11].

The per cent citral indicated in Table III differs from that reported in the experimental section above. The value reported in the experimental account was determined by reaction with hydroxylamine (the common method employed in the citrus industry) and actually reflects the total aldehyde and ketone content of the oil. The percentage reported in Table III more nearly represents the citral content exclusive of other aldehydes and ketones that are present in the oil.

## ACKNOWLEDGEMENT

The author is indebted to the Sunkist Growers, Inc. of Los Angeles, Calif. for their generous aid in this research.

## SUMMARY

Cold-pressed California lemon oil was examined by means of gas–liquid chromatography and a number of the constituents present was tentatively identified by means of corrected retention volume correlation employing two stationary liquid phases. The components identified by these procedures were: α-pinene, β-pinene, myrcene, *d*-limonene, *n*-heptanal, 1,8-cineole, γ-terpinene, *n*-hexanol, *n*-octanal, methyl heptenone, *n*-nonanal, linalool, *n*-decanal, citronellyl acetate, citronellal, *n*-undecanal, tetrahydrogeraniol, decyl acetate, geraniol, α-terpineol, citronellol, neral, geranyl acetate, citral, and *d*-carvone.

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# CONTINUOUS ELECTROPHORETIC SEPARATIONS OF RADIOACTIVE RARE EARTH MIXTURES

## I. SEPARATION OF $^{144}$Ce–$^{160}$Tb–$^{170}$Tm AND $^{144}$Ce–$^{152}$Eu–$^{169}$Yb IN 0.05 *M* LACTIC ACID

Z. PUČAR AND Z. JAKOVAC

*Institute Rudjer Bošković, Zagreb, Croatia (Yugoslavia)*

(Received November 23rd, 1959)

### INTRODUCTION

The continuous electrophoretic separations of mixtures of radioactive inorganic ions were first described by STRAIN *et al.*[1-3]. They successfully separated mixtures such as $^{45}$Ca–$^{32}$PO$_4^{3-}$ in 0.1 *M* lactic acid or 0.1 *M* acetic acid[1], which served as basic electrolytes, $^{90}$Sr–$^{90}$Y in 0.1 *M* lactic acid[2], $^{90}$Sr–$^{90}$Y–$^{32}$PO$_4^{3-}$, $^{45}$Ca–$^{90}$Y–$^{32}$PO$_4^{3-}$, $^{90}$Sr–$^{90}$Y–$^{144}$Ce–$^{32}$PO$_4^{3-}$, $^{137}$Cs–$^{90}$Sr–$^{90}$Y–$^{60}$Co–$^{32}$PO$_4^{3-}$, and $^{137}$Cs–$^{90}$Sr–$^{90}$Y–$^{233}$U–$^{95}$Zr–$^{95}$Nb also in 0.1 *M* lactic acid[3]. A continuous electrophoretic separation of radioactive Ac from inactive La has been published by LEDERER[4].

In the present series, the continuous electrophoretic separations of radioactive rare earth mixtures on filter paper are described. Filter paper serves as a supporting and anticonvection medium, and at the same time shows the properties of an adsorptive capillary system. Considering the differences that exist between the continuous and discontinuous separation process occurring on an adsorptive supporting medium, which have been discussed in detail earlier[5,6], the parallel treatment of the discontinuous or two-dimensional electrochromatographic separations will be considered in the present paper.

### EXPERIMENTAL

The apparatus used for continuous electrophoresis and for the discontinuous two-dimensional electrochromatography was described earlier[5]. The electrodes, consisting of Pt wires, are placed along the sides of a filter paper curtain, and are rinsed continuously with a solution of the basic electrolyte. The filter paper curtain is stretched freely between the two electrode channels in the wet chamber without cooling. Thus our apparatus represents a modification of GRASSMANN AND HANNIG's apparatus[7]. The mixture to be separated is pumped continuously at a controlled rate by means of an electrically driven syringe*.

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* Manufactured by Bender & Hobein, Munich, Germany.

The experimental conditions for the separations of the two rare earth mixtures, Ce–Tb–Tm and Ce–Eu–Yb, were the same, and so the results can be compared. The rare earths were first dissolved in nitric acid, evaporated to dryness, and then dissolved in 0.05 *M* lactic acid. The basic electrolyte was a 0.05 *M* solution of lactic acid. The filter paper used was Munktell No. 20/250. The distance between the electrode channels, *i.e.* the free width of the filter paper curtain was 300 mm, and the distance between the starting point and the lower edge of the paper, *i.e.* the free height of the curtain was 320 mm. The vertical speed of flow of the basic electrolyte, *i.e.* the time interval between the input and the outlet of the substance to be separated was 2¾ h.

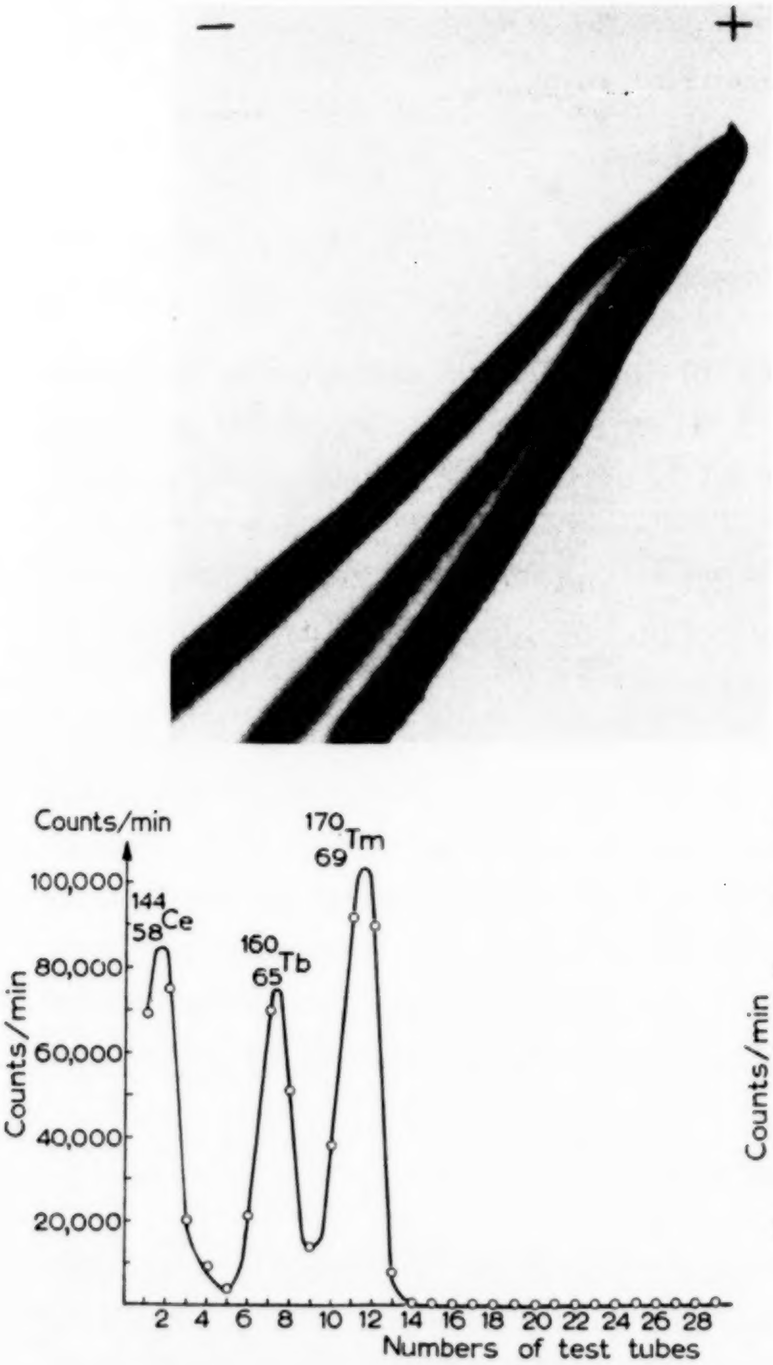

Fig. 1. Radioautograph of the continuous electrophoretic separation of $^{144}$Ce–$^{160}$Tb–$^{170}$Tm. The diagram below represents the activities of separated rare earths which were detected in the collecting glasses. Collecting time was 0.5 h. Electrolyte, 0.05 *N* lactic acid; paper, Munktell No. 20/250; voltage drop, 300 V; mean electrical field strength, 10 V/cm; current, 19 mA; pumping rate, 0.2 ml/h.

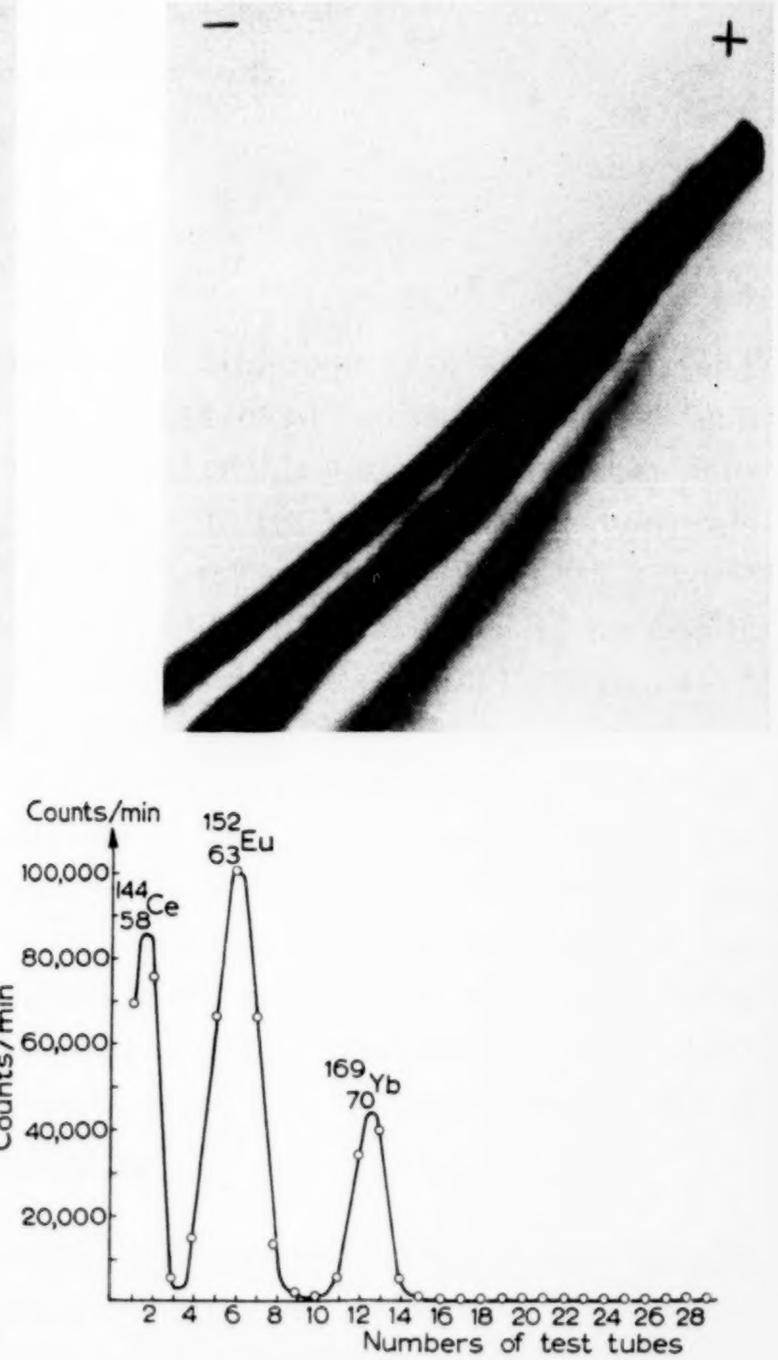

Fig. 2. Radioautograph of the continuous electrophoretic separation of $^{144}$Ce–$^{152}$Eu–$^{169}$Yb. The diagram below represents the activities of separated rare earths which were detected in the collecting glasses. Collecting time was 0.5 h. Electrolyte, 0.05 *N* lactic acid; paper, Munktell No. 20/250; voltage drop, 300 V; mean electrical field strength, 10 V/cm, current, 19 mA; pumping rate, 0.2 ml/h.

The electrode channels were sealed with a cellophane tape of ¾ in. width, which acted as a membrane[6]. The voltage drop between the electrodes was 300 V, the mean electrical field strength being 10 V/cm, and the current 19 mA. The pumping rate of the rare earth solution was 0.2 ml/h.

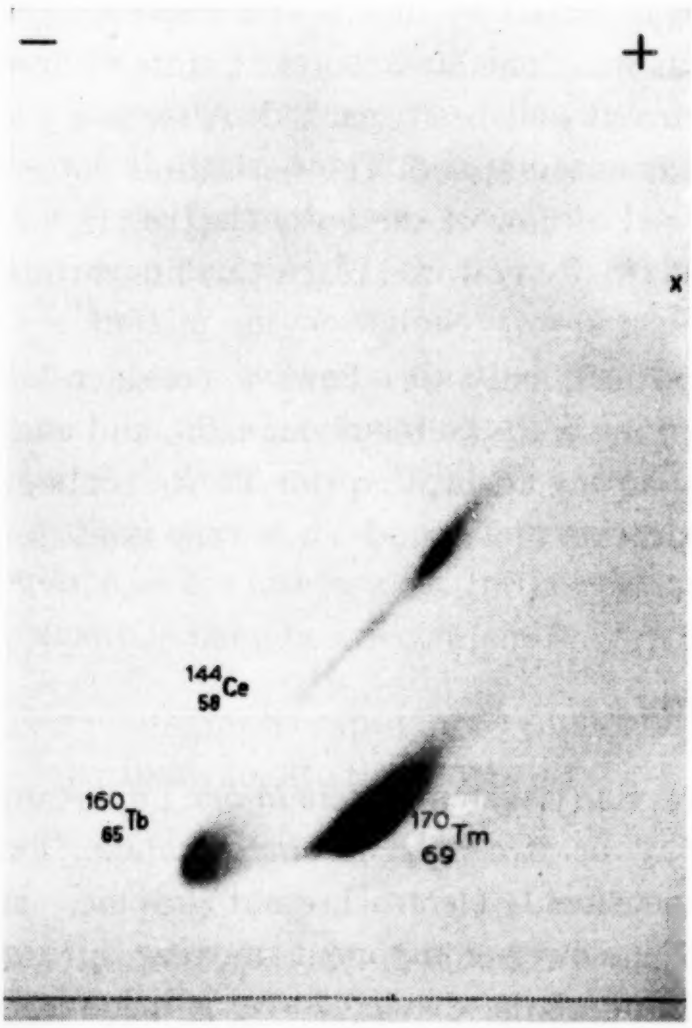

Fig. 3. Radioautograph of the two-dimensional electrochromatographic separation of $^{144}$Ce–$^{160}$Tb–$^{170}$Tm. Spots with no adsorption on the filter paper would reach the horizontal dotted line during the experiment.

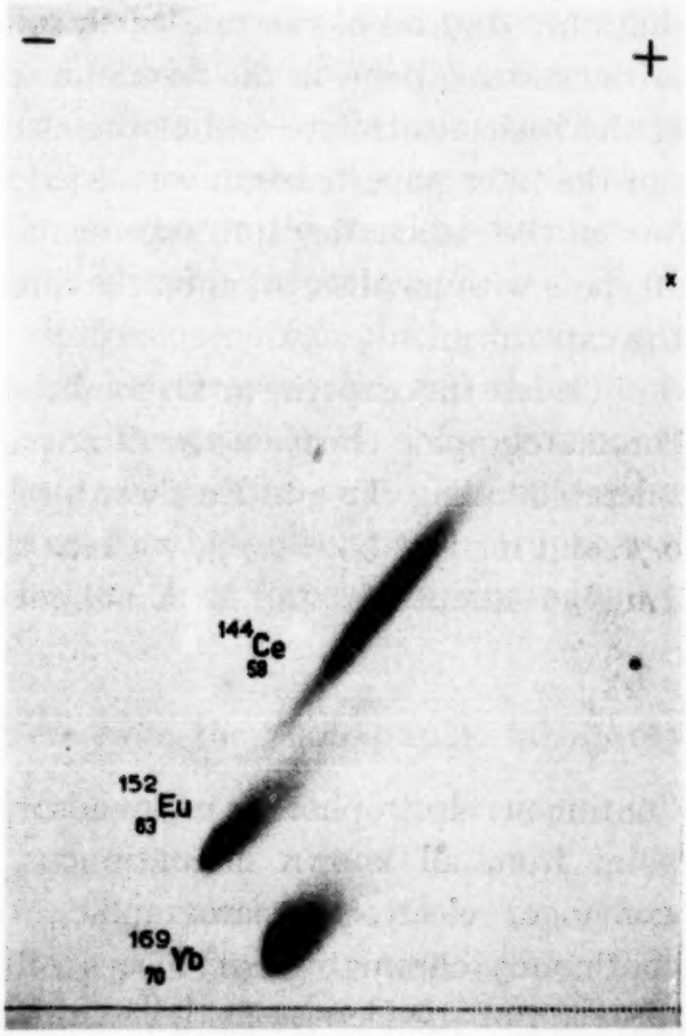

Fig. 4. Radioautograph of the two-dimensional electrochromatographic separation of $^{144}$Ce–$^{152}$Eu–$^{169}$Yb. Spots with no adsorption on the filter paper would reach the horizontal dotted line during the experiment.

The stationary state of the continuous separation process was reached after 5 h of operation, when all the components of the rare earth mixtures left the apparatus in a steady flow equal to the input pumping rate. A new set of collecting tubes was then introduced for a period of 30 min and the solutions evaporated on aluminium discs under an infrared lamp. The radioactivity of the aluminium discs was then measured by means of a Geiger-Müller counter with a mica window. To prepare the radioautographs, the filter paper curtain was dried in a stream of hot air, laid in a cellophane bag, and then exposed to a "Supervidox"* X-ray film, 30 × 40 cm for about 16 h.

Figs. 1 and 2 represent radioautographs of the continuous separations of the mixtures $^{144}$Ce–$^{160}$Tb–$^{170}$Tm and $^{144}$Ce–$^{152}$Eu–$^{169}$Yb in 0.05 *M* lactic acid. Below each of the radioautographs there is a diagram showing the activities of the separated rare earths detected in the dried material from the collecting glasses. These activities correspond to a 0.5 h run, *i.e.* to 0.1 ml of the initial solution of the rare earths used.

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* Manufactured by VEB Fotochemische Werke, Berlin.

The radioautographs of the discontinuous two-dimensional electrochromatographic separations of the two mixtures are represented in Figs. 3 and 4. All the experimental conditions were the same as for the continuous separations, except that only 0.02 ml of the rare earth solution was placed by means of a micropipette at the starting point in the form of a spot. This was done after a steady state of flow of the basic electrolyte and of the electric current had been reached. After a 2.5 h run the filter paper curtain was dried and radioautographed. The horizontal dotted line on the radioautographs represents the speed of flow of the basic electrolyte, *i.e.* all spots with no adsorption on the filter paper ($R_F = 1$) would reach this line during the experiment.

Under the experimental conditions described, only Ce shows a considerable chromatographic effect on the filter paper, having a $R_F$ factor about 0.60, and considerable tailing. Tb and Eu show a relatively strong adsorption (for Tb $R_F$ is about 0.78 and for Eu about 0.79), whereas the adsorption of Tm and Yb is very faint (for Tm $R_F$ is about 0.82 and for Yb about 0.95).

## DISCUSSION

Continuous electrophoresis on an adsorptive capillary system differs in one important point from all known discontinuous one-way or two-way chromatographic, ion exchange, electrochromatographic, two-dimensional electrochromatographic, or continuous chromatographic separations: the slower component moving in the direction of the electric field, *i.e.* in the direction of the driving force, is in no way contaminated by the reversibly adsorbed fraction of the faster moving component during the continuous electrophoretic separation process. Particles or ions, which show different electrokinetic mobilities, follow separate tracks in the continuous electrophoretic process, and thus absolute separations can be achieved. This is of particular importance when an absolute separation of radioactive ions of high specific activities is desired. Moreover, by the continuous electrophoretic method, carrier-free preparations are possible even from a very dilute solution of a particular ionic species, and even though other ions may be present in considerable concentrations.

Although continuous electrophoresis is primarily considered to be a method for preparative separations, qualitative and quantitative analyses can in principle be readily carried out. This method can be used as an especially convenient analytical tool, in the case of the separation of radionuclides. When the stationary state of the continuous process is once reached, *i.e.* when all the radionuclides to be separated leave the apparatus in a steady flow, a new set of collecting glass tubes should be introduced for a known time. If the pumping rate of the continuously applied test solution is known, radiometric qualitative and quantitative analyses of the radionuclides from the collecting tubes can readily be performed in the usual manner.

A further advantage of continuous electrophoresis used for separating hazardous radioactive materials lies in the fact that the process can be maintained fully automatically with relatively small expense.

According to the theory[5], in the two-dimensional electrochromatographic technique the filter paper acts as an adsorptive capillary system. The two-dimensional discontinuous separation is a result of two simultaneous separations acting at right angles: the horizontal or electrochromatographic, and the vertical or chromatographic separation. The characteristics of the two-dimensional electrochromatographic separation process are the following: (1) particles travelling on the same track have identical electrophoretic mobilities; (2) particles travelling on different tracks have different electrophoretic mobilities; (3) particles moving in the vertical direction on the same horizontal front have the same chromatographic properties ($R_F$).

In the discontinuous two-dimensional electrochromatographic process, only those components of a mixture that show identical electrophoretic, and at the same time, identical chromatographic properties are inseparable. In the continuous electrophoretic process, which may, or may not, be carried out in an adsorptive capillary system, all those components of a mixture that have identical electrophoretic mobilities are inseparable. In the continuous electrophoretic process chromatographic properties have no effect upon the separation.

The photographs reproduced in this article were from the photographic laboratory of the Institute Rudjer Bošković.

## SUMMARY

Radioautographs of the continuous electrophoretic separations and of the two-dimensional electrochromatographic separations of two mixtures, $^{144}$Ce–$^{160}$Tb–$^{170}$Tm and $^{144}$Ce–$^{152}$Eu–$^{169}$Yb, in 0.05 $M$ lactic acid are given. Below each radioautograph of the continuous separation, the activities of the separated rare earths are presented in a diagram. These radioactivities detected in the collecting glasses correspond to a 0.5 hour run. From the diagrams of activities and from the radioautographs it is evident that the separation of both rare earth mixtures into components was complete.

The radioautographs of the discontinuous two-dimensional electrochromatographic separations show a relatively strong adsoprtion of Ce, Tb and Eu on the filter paper. These elements give spots with comet-like tailings.

The usefulness of the continuous electrophoretic separations of radionuclides for preparative carrier-free separations and radiometric qualitative and quantitative analyses is discussed.

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# QUALITATIVE, QUANTITATIVE AND PREPARATIVE CHROMATOGRAPHY OF STEROIDS ON FULLY ACETYLATED PAPER

## II. QUANTITATIVE CHROMATOGRAPHY OF $\Delta^4$- AND $\Delta^{1,4}$-3-KETOSTEROIDS*

J. HARTEL, A. BOL RAAP AND F. J. RITTER
*Central Laboratory T.N.O., Delft (The Netherlands)*

(Received August 31st, 1959)

### INTRODUCTION

Various methods are known for quantitative paper chromatography of weakly polar steroids. Use is often made of impregnated paper in order to ensure proper separation, although this involves drawbacks of various kinds. Upon elution of the spots after chromatography, the solution to be measured also often contains the impregnating substance, which may adversely affect spectrophotometric determination through causing high, irregular paper blanks. As these impregnating substances are often nonvolatile, they are difficult to remove[1]. Also in colorimetry, in which steroids are converted into coloured compounds, the impregnating substance may have a disturbing effect.

If untreated paper is used for chromatographic separation, adsorption of steroids on the paper may cause substantial losses during development of the chromatogram and during elution of the spots[2].

These difficulties can be obviated by using fully acetylated paper. A method of qualitative separation on this paper has already been developed by us[3] and in a preliminary communication we stated that this method could also be used for quantitative and preparative purposes[4]. The present paper describes the quantitative determination more fully. Owing to there being no impregnating agent and because steroids are virtually not adsorbed on acetylated paper, a high degree of accuracy was obtained.

### METHODS

### *A. Papers*

A strip of untreated Whatman No. 4 paper, 7 × 17 cm, was sewn, with a 1 cm overlap, to a sheet of Whatman No. 1 paper, 44 × 17 cm, fully acetylated in our laboratory (ZIJP's method[5], slightly modified[3]), in order to retard the mobile phase.

The paper was then chromatographically washed by the descending method with 100 ml methanol p.a.* and afterwards dried for 10 min at 80°. A strip 1.5 cm wide was cut from both long sides of the paper, as impurities often remain in the edges. The length of the retardation strip was then decreased 4 cm. The starting line was marked 5 cm from the bottom of this strip, *i.e.* 3 cm from the bottom of the acetylated paper. The sheet was then divided into five "runs" with pencil lines.

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* Part I: Qualitative chromatography of $\Delta^4$- and $\Delta^{1,4}$-3-ketosteroids, *J. Chromatog.*, 1 (1958) 461.

### *B. Application of the steroids to the paper*

On the starting line two spots were applied of a solution of the steroid mixture for investigation and two spots of a standard solution of the steroid to be determined.

The aim was a dosage of 80–120 $\mu$g of the steroid in 80 $\mu$l of methanol. If the quantity of steroid in the mixture was not known approximately, a trial chromatogram was made first. For this purpose varying quantities of a solution of the mixture were employed beside a known quantity of the steroid to be determined. After chromatography the spots were compared and the concentration of the steroid in the solution of the mixture was estimated.

Dosing was done by means of an Agla micrometer syringe** in sixteen fractions of 5 $\mu$l each, which were meanwhile blown dry with nitrogen in order to keep the spots small. One of the five runs on the sheet was left open for determination of the paper blank (see D).

### *C. Chromatography*

After application of the spots, the acetylated paper was hung in a chromatography cylinder (19 cm in diam.; 50 cm in height) and accommodated for 16 h at 23° over 120 ml of the lower layer (with high benzene content) of the liquid mixture: benzene p.a.–methanol p.a.–water (4:4:1). Next the chromatogram was developed for 8 h with the upper layer (with high methanol content) by the ascending technique (method B, see[1]). The chromatogram was then dried at 60° for 5 min.

The spots can be marked under a "Chromatolite" lamp*** (max. 253.7 m$\mu$), as they are visible as dark spots in U.V. light of this wavelength. In the brief time required for this marking, no photochemical conversion or attack could be found.

### *D. Elution*

The slightly oval spots were cut out with a small margin so that one of the short sides became pointed and the other was folded over for a length of about 3 mm. This edge was held between two glass slides placed at an incline in a porcelain dish filled with methanol p.a. (DENT's elution method[6]). As the acetylated paper is fairly thick, the distance between the two slides was too great for adequate capillary attraction of methanol. By grinding one of the slides at the place where the paper lies, however, the space between the slides was made small enough. The strip of paper hung point downwards over a special 10 ml calibrated, pear-shaped distillation flask; 7 to 8 ml eluate was collected. (It was found experimentally that a spot of 100 μg cholestenone with 7 ml methanol could be 100 % eluted from the acetylated paper. A similar test with non-acetylated Whatman No. 1 paper, however, showed an 8 % loss.)

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* Methanol pro analysi, und für die Chromatographie. Merck.

** Burroughs Wellcome Co., London.

*** Hanovia Ltd., Slough, England.

A similar piece of paper was cut from the control run at the same height as the spot being determined and was likewise eluted. The eluate may contain traces of benzene from the chromatogram. As benzene disturbs spectrophotometric determination it was removed by evaporation in vacuum under nitrogen. A simple unit was built to which three measuring flasks can be connected at once (Fig. 1). The residue was made up to 10 ml with methanol p.a.

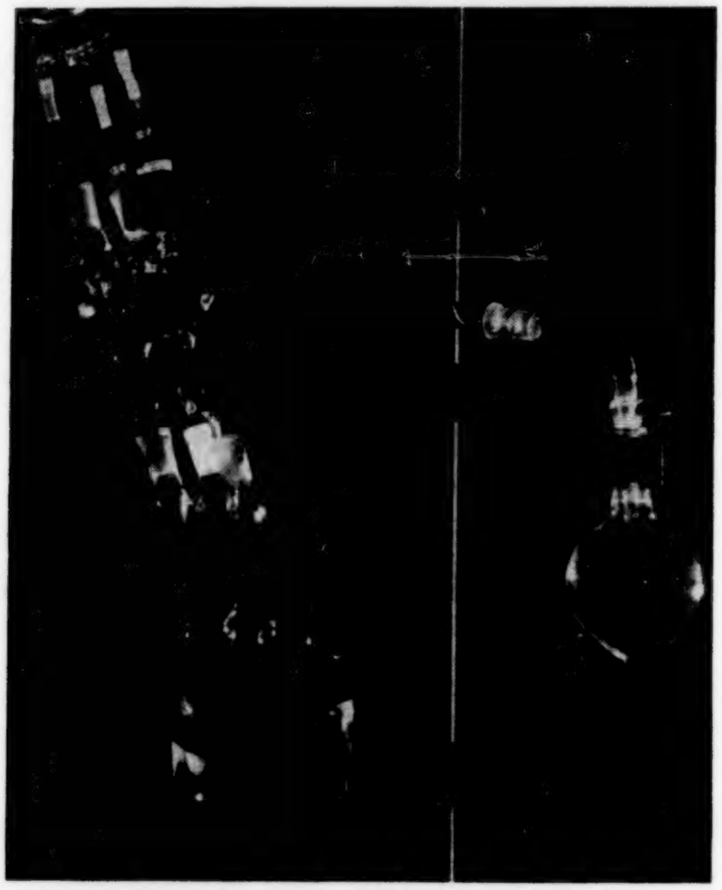

Fig. 1. Apparatus for combined distillation from three calibrated measuring flasks.

### *E. Spectrophotometric determination*

Before the solutions obtained were measured in a spectrophotometer*, the calibration curve of the steroid was determined by measuring two methanolic solutions of the steroid in various dilutions against methanol. Quartz glass cuvettes** were used, while measurement took place at the $\lambda_{\text{max.}}$ of the steroid. For cholestenone we found 242 mμ, for progesterone 240.5 mμ, for testosterone 241 mμ, for hydrocortisone 241.5 mμ and for prednisolone 243 mμ.

The extinction values were then determined for the solutions obtained after chromatography and elution. The value found for the paper blank was deducted.

Losses that might arise through chromatography were determined by measuring the chromatographed and eluted standard spots. These losses were found to be approximately proportional to the quantities applied (Fig. 2).

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* Unicam spectrophotometer SP 500.

** Quartz glass Ultrasil.

To find any possible error in dosage, the same volume of the standard solution that was applied to the paper, was also added to a measuring flask with the aid of the same syringe and was subsequently made up to 10 ml with methanol. By determining the extinction of this solution, it was ascertained via the calibration curve how much steroid had really been applied to the paper.

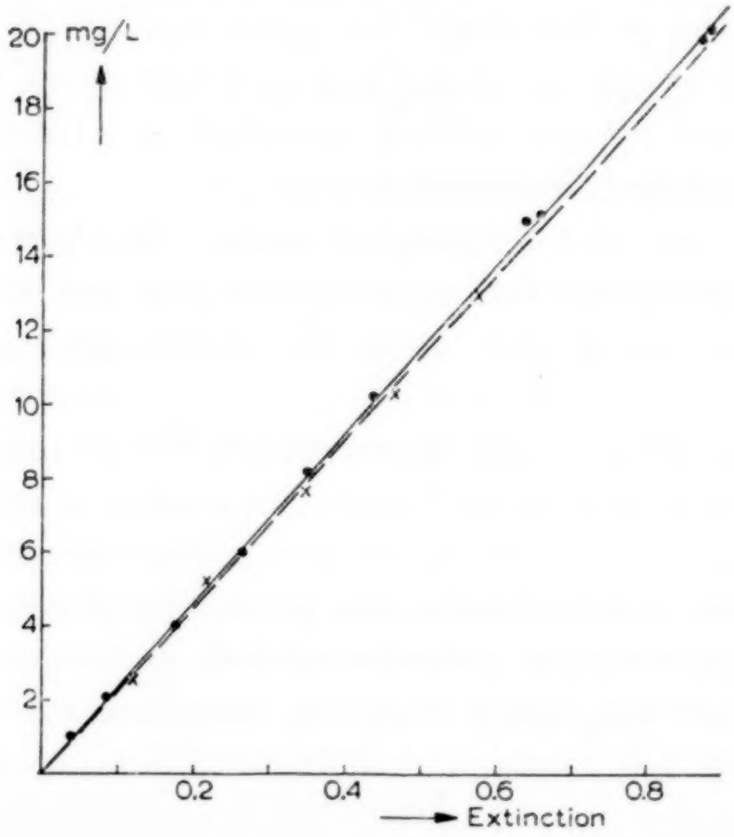

Fig. 2. Cholestenone extinctions determined at 242 mμ: —— calibration curve obtained with standard solution (● ●); – – – calibration curve obtained with chromatographed standard (× ×).

The corrected extinction value of the steroid that is to be determined can then be calculated with the formula:

$$a = \frac{c}{b - d}(e - d)$$

in which: $a$ = corrected extinction of the steroid,

$b$ = measured extinction of the solution of the steroid chromatographed and eluted from the standard solution,

$c$ = measured extinction of solution of a similar quantity of steroïd not chromatographed,

$d$ = measured extinction of solution obtained after elution of the paper blank from the control strip,

$e$ = measured extinction of solution of the steroid to be determined after chromatography and elution.

With the aid of the calibration curve and value $a$ the steroid concentration can be ascertained.

## RESULTS

The method was tried out with the following steroids: $\Delta^4$-cholesten-3-one, progesterone, testosterone, hydrocortisone and prednisolone.

The accuracy of the method was ascertained for cholestenone and progesterone by means of twenty determinations. For each of these substances four strips of paper were used as described in A. Per sheet, five spots were applied in quantities of 26, 52, 78, 104, 130 µg and 25, 49, 74, 99 and 124 µg, respectively. Chromatography and elution were carried out by the method described and the concentration of the solutions was determined spectrophotometrically.

The results show that with a dosage of approx. 25 µg the errors are greatest. Spots of 50–125 µg had about the same relative standard deviation. With sixteen determinations (50–125 µg) it was 4.0% for cholestenone and 3.8% for progesterone.

For testosterone a total of nine determinations were made, *i.e.* in dosages of 49 µg (three), 73 µg (three) and 97 µg (three). The relative standard deviation in this case was 4.9%.

Random tests with hydrocortisone and prednisolone also indicated that these steroids can be determined by the method described.

Lastly, this method was tested with two trial mixtures of cholestenone and

TABLE I

| | *Extinction (av.)* | *Quantity* | *Percentage of steroid in mixture* | |
|---|---|---|---|---|
| | | | *as found* | *actual* |
| *Mixture I* | | | | |
| Cholestenone determination | | | | |
| Paper blank | 0.038 | | | |
| Stand. soln. | 0.428 | | | |
| Stand. soln. after chrom. | 0.438 | | | |
| 134 µg mixture | 0.436 | 99 µg | 74 | 73 |
| Progesterone determination | | | | |
| Paper blank | 0.038 | | | |
| Stand. soln. | 0.524 | | | |
| Stand. soln. after chrom. | 0.514 | | | |
| 309 µg mixture | 0.458 | 86 µg | 28 | 27 |
| *Mixture II* | | | | |
| Cholestenone determination | | | | |
| Paper blank | 0.050 | | | |
| Stand. soln. | 0.428 | | | |
| Stand. soln. after chrom. | 0.460 | | | |
| 324 µg mixture | 0.331 | 68 µg | 21 | 22 |
| Progesterone determination | | | | |
| Paper blank | 0.038 | | | |
| Stand. soln. | 0.524 | | | |
| Stand. soln. after chrom. | 0.570 | | | |
| 141 µg mixture | 0.633 | 109 µg | 77 | 78 |

progesterone. Two paper strips were used for each mixture, one for cholestenone and one for progesterone. 20.628 mg of mixture I and 21.635 mg of mixture II were each dissolved in 10 ml methanol.

The results are given in Table I.

The absolute error in the determined percentage of the steroid in the mixture in all four cases was found to be only about 1%.

## DISCUSSION

Chromatographic washing of acetylated paper with methanol proved to be necessary in order to obtain low and only slightly varying blanks.

During dosage of the steroids, losses may occur through the solution creeping along the syringe. By grinding the needle square and holding it at an angle to the paper during application of the solution, the loss can be kept very low and virtually constant.

The chromatogram is dried at low temperature to obviate any losses through atmospheric oxidation.

The elution method employed is more satisfactory than that with which the paper strips are shaken in solvent[1], as this leaves fine paper fibres floating in the solution.

The presence of benzene in the eluate causes high and greatly varying control values owing to the steep absorption peaks of benzene in the measuring range. As intensive drying of the paper did not suffice to remove the benzene quantitatively, the eluate was dried by evaporation *in vacuo*.

Use of the same combined distilling and measuring flask for collecting and evaporating the eluate and subsequent dissolving in methanol in order to determine the extinction obviated decanting losses.

## ACKNOWLEDGEMENT

The authors express their sincere thanks to Dr. G. J. SCHURINGA for his encouragement.

## SUMMARY

A method has been developed for quantitative chromatography of $\Delta^4$- and $\Delta^{1,4}$-3-ketosteroids on fully acetylated paper. The lack of an impregnating agent and the very slight adsorption of these steroids on acetylated paper made exact determination possible. The standard deviations of cholestenone, progesterone and testosterone were determined, while several tests were made with prednisolone and hydrocortisone.

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# Short Communications

## Spotting apparatus for the application of large quantities of fluids on paper chromatograms*

### *Introduction*

The apparatus to be described represents the final product of an original design presented elsewhere four years ago[1]. The present prototype unit was constructed for us and according to our specifications by the "Gilson Medical Electronics"**. It is designed to facilitate the application of biological and other fluids in a small spot of filter paper for chromatographic analysis. Following our original report[1], VAN GULIK[2], LEVENBROOK[3], DU RUISSEAU[4], VAN DER SIJDE AND DE FLINES[5] described also spotting devices which we believe lack the efficiency of our present unit particularly so far as the size of the spot is concerned and the turnover of multiple specimens.

### *The apparatus*

The basic principle of the apparatus is transfer of fluid specimens from a set of eight tuberculin syringes (5 mm bore, Ideal East Rutherford Syringe Inc.) by continuous slow ejection onto chromatographic paper while the air pressure is reduced under the area spotted by means of an adjustable vacuum pump mechanism. The latter provides for fast evaporation of the drops ejected on the paper so that the dry material deposited covers a small uniform area at all times. Fig. 1 is an exploded diagram of the basic features of the apparatus, while the completed form of the spotting unit is presented in Fig. 2. Each syringe (H) is secured in place by the upper (D) and lower (J) syringe holder plates mounted steady in the device by means of mounting rods (F). The upper plate provides 8 notched clamps (E) to support the upper part of the syringes. The lower syringe holder-plate carries the height adjustment clamps (K) to secure contact of the syringe tips to the chromatographic papers. The fluid expelled from each syringe is applied to the paper through a short length (1 mm bore) polyethylene tubing held tight inside the tip of the syringe. When this unit is in operation, the plungers of the syringes are pressed uniformly by the plunger depressor plate (C) driven downwards by means of the center axis (A). The latter is operated by a three speed Bodine synchronous motor (type NCH-13R) designed to operate as a two pole, four pole, or eight pole motor which will make the plunger plate move respectively 1.6, 3.2, or 6.4 cm per hour. This will correspond to ejection of about 0.3, 0.6 and 1.2 ml of fluid per hour in respective order. A pressure equalizing stud (B) for each plunger

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** Gilson Medical Electronics are located at Middleton, Wis., U.S.A.

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permits the plunger plate to travel safely a short distance after the syringes are completely empty at which time the ejecting motor is automatically turned off. In addition to the mechanism for slow downward movement of the plungers, a separate motor (Bodine, speed reduced motor type NSY-12R) is provided for fast raising and lowering of the plunger plate by way of the center axis. The latter aids in the prepara-

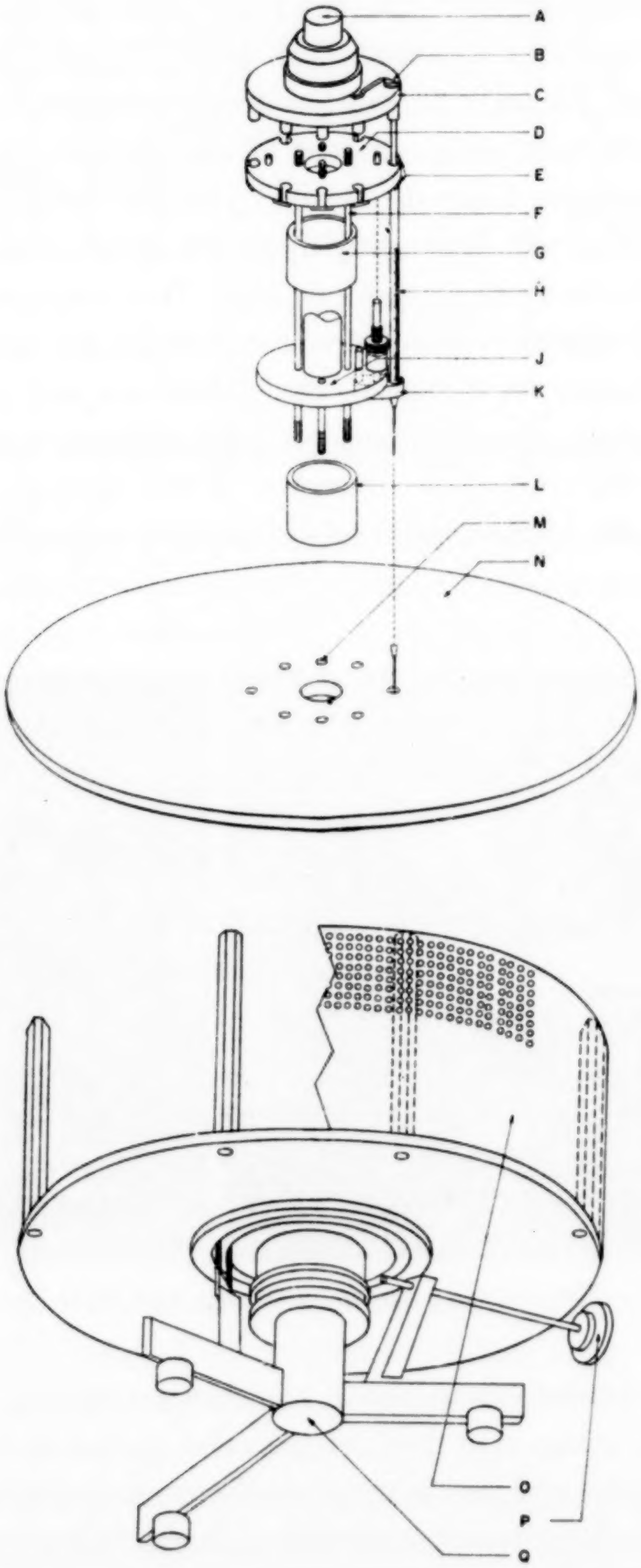

Fig. 1. Partially exploded diagram of the spotting apparatus. A. Center axis; B. Pressure equalizing stud; C. Plunger depressor plate; D. Upper syringe-holder plate; E. Supporting clamp-notch; F. Mounting rod; G. Spacer; H. Tuberculin syringe; J. Lower syringe-holder plate; K. Height adjustment clamp mechanism; L. Spacer; M. Suction opening; N. Spotting platform; O. Body of apparatus; P. Brake arm; Q. Supporting tripod.

tion of the unit for operation. The entire syringe holding system is centered above the body (O) of the machine containing the motors and vacuum pump which are not shown in the figures. The top plate (N) of the housing unit has 8 suction openings (M) of 5 mm diameter each corresponding to the tips of the syringes. Each opening communicates with a closed system of reduced pressure induced by a rotary vacuum pump and compressor unit (Gast Manufacturing Corporation 0321-V2G18K) for rapid evaporation of the solution applied onto the filter paper above the opening. The lip of each opening projects 2 mm above the top plate (N) and has a slightly irregular surface to prevent high vacuum formation from complete contact of the paper around the opening. The compressor motor of the vacuum pump is automatically turned off when the syringes have discharged the last drop of fluid. The negative pressure can be adjusted at will depending upon the speed of the ejection chosen, the volatility of the solvent, and size of spots desired. The entire apparatus is supported by a heavy tripod (Q) designed to permit free rotational movement of the unit checked by brake arm (P) for easier handling of the specimens and papers processed. The panel of control switches, pressure gauge, and gear systems are conveniently located at the front of the unit. The diameter of the syringe supporting system is 7 ½ in. while the diameter of the main body measures 25 in. The total height of the apparatus is 26 in.

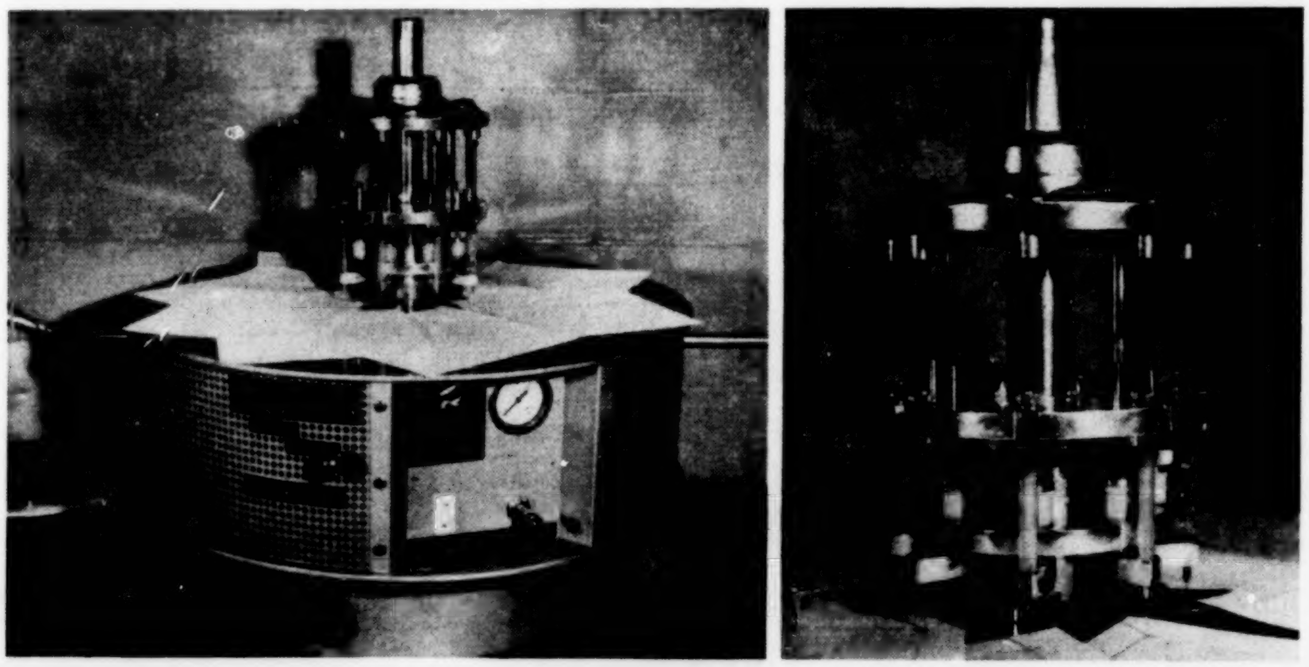

Fig. 2. The spotting apparatus in its completed form.

For body fluids and solutions in water, the slow or medium speeds of ejection are best for accurate work using 2 to 3 in. of negative pressure respectively. For more volatile solvents fast speed and less negative pressure are sufficient. With such speeds and pressure the total diameter of the spot is kept under half a centimeter. The efficiency of the unit was tested with solutions of radioactive phosphorus ($^{32}P$) for possible losses of solutes by the continuous application of suction under the spot. It was found that with the pressure mentioned above, there is absolutely no loss of radioactive phosphorus.

The author expresses appreciation to Dr. W. E. GILSON for his valuable assistance in the mechanical perfection of the present apparatus.

*University of Minnesota Medical School,*
*Minneapolis, Minn. (U.S.A.)*
JOHN LOGOTHETIS

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# Apparatus for collecting and drying organic acid fractions

Papers on the ion-exchange fractionation of organic acids have involved the use of commercial fraction collectors designed for collecting fractions in 18 × 150 mm test tubes[1-3]. These papers describe methods for evaporating the fractions in the test tubes in which they are collected. Evaporation is necessary in order to remove the volatile "swamping acids" used for elution before titrating the non-volatile residues. However, evaporating liquids in test tubes is difficult because of the low surface to volume ratio of the liquid, the long path the vapor must travel before leaving the tube, and the tendency of vapor to condense on the upper part of the tube. Test tubes are also inconvenient as titration vessels. The following describes a device which can be used on a Technicon Fraction Collector* for collecting 200 fractions in 25 (O.D.) × 50 mm shell vials and also simple methods and devices for evaporating and titrating the fractions.

*Fraction collector attachment.* Since the holes for inserting test tubes in the Collector Rack** were too close together for vials to be placed in the corresponding positions, a new pattern had to be made. It was found that to accommodate as many vials as there had been test tubes, the vials in the innermost circle would have to touch each other and those in the outermost circle be very close to the rim of the collector housing. This necessitated the vials being placed in four circles having radii of 20, 22.5, 25 and 27.5 cm instead of the normal 17.8, 19.7, 21.9 and 24.1 cm.

A disk 28.7 cm in radius was cut from 1.3 cm waterproof plywood and on it were drawn four circles having the above radii. Knobs were attached to act as handles,

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* The mention of companies and commercial products in this paper does not imply that they are endorsed or recommended by the Department of Agriculture over others not mentioned.

** Capitalized names refer to the Technicon fraction collector or to parts of it as named in the Technicon instruction manual.

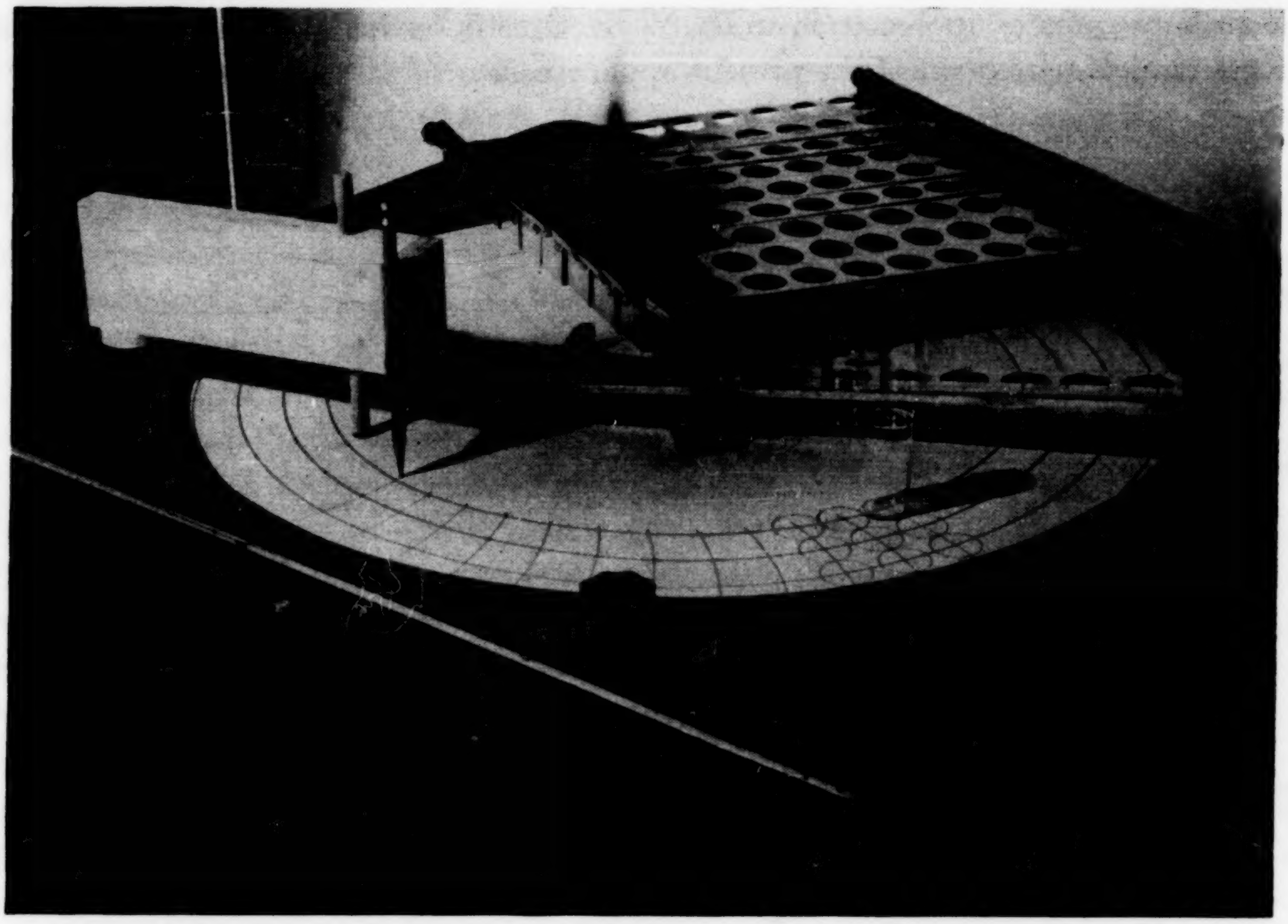

Fig. 1.

and the disk was attached to the Collector Rack by means of two screws. It was necessary to cut a narrow strip of metal from the back side of the Collector housing rim (heavy shears), because the Rack was not centered exactly under the housing opening. Dimensions are critical and should be checked against the individual Technicon before proceeding.

The Funnel Support Arm was replaced by a board drilled at one end to fit over the pins on the Funnel Arm Post and bearing on the other end a board attached at right angles to it and slotted to support a pencil in the vertical position (see Fig. 1). The latter board compensated for the greater radii of the circles of vials as compared with the circles on the Collector Rack. A dowel held in a hole drilled vertically through the board (friction fit) acted as a bearing to support the free end of the board. A thumb tack on the bottom end of the dowel allowed it to slide easily. The distance from the Post to the pencil (about 19.5 cm) should be such that at each position of the board (corresponding to positions of the Funnel Support Arm) the pencil is over one of the circles drawn on the disk. After ascertaining this, a wooden washer was placed under the board so that it could swing freely. Then, while operating the Technicon, the board and pencil attachment was used to draw arcs which intersected the concentric circles at the vial positions. The disk was removed from the Rack and 2.6 cm diameter, 1 cm deep, pits were drilled at these positions. The disk was then sprayed with an acid-resistant paint (thin coats!).

The Dropping Funnel was lengthened and bent so that its tip had the same position as the pencil and was over the center of the pits at the various positions of the Collector Rack and the Funnel Support Arm.

The plywood disk worked satisfactorily, but some difficulty was presented by uneven thickness of the paint. There is also the possibility of future warping. A more durable, though more expensive, attachment for vials was made from 1.3 cm thick aluminum plate. This had as much of the center cut out as possible in order to reduce weight.

*Evaporating and titrating fractions.* Racks, each holding a row of ten vials, were made from two strips of aluminum sheet. The bottom strip was bent up at right angles at each end, and the top strip, containing holes for the vials and bent down along the sides for added strength, was attached to it by the tongue and slot method. The upright ends of the bottom strip were bent back to form slots through which rods could be passed holding the racks in groups of ten.

Drying chambers were made from four 48 × 41 cm pieces of plate glass (roof and floor of chambers) and three 43 × 8 × 4.5 cm blocks of wood (sides and central support). The chambers were set up in the front part of a fume hood, the hood window closed down to the chamber roof, and openings at the sides of the chambers were blocked off. This arrangement dried 200 1 ml-fractions in about 8 hours at room temperature. To increase evaporation rate a sheet-metal box containing two 500-W Chromalox air heaters was built. This, when placed in front of the drying chambers, raised the air temperature a few degrees and cut the drying time to 4 hours.

After the drying step the racks were separated by removing the connecting rods and the fractions titrated in groups of ten. One ml water and a micro stirring bar (Macalaster Bicknell Company, Millville, New Jersey) was placed in each vial. A strip of aluminum sharpened along one edge and painted white was slipped under the vials. The vials were stirred during titration by passing the rack over a magnetic stirrer. The stirring bars were easily transferred from one set of vials to the next by the use of a magnetized "pick-up" stick.

*Acknowledgements.* The author wishes to express his appreciation for help received from personnel of the shops and other divisions of the laboratory.

*Eastern Regional Research Laboratory*, Agricultural Research Service,* J. H. SCHWARTZ
*United States Department of Agriculture, Philadelphia, Pa.* (*U.S.A.*)

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* Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture, Philadelphia 18, Pa.

# Relative detector response in gas chromatography
## II. Benzene hydrocarbons, phenols and phenol ethers

In a previous communication[1] it was shown that, in a homologous series there is a relationship between relative detector response and molecular weight. The object of the present work was to investigate the relative detector response to isomeric benzene derivatives to find if the relative detector responses altered

(a) with the relative positions of substituents attached to the benzene ring, and

(b) when the substituent was attached to the benzene ring or when it was attached to a side chain.

MESSNER *et al.*[2], using helium as the carrier gas, found that ethylbenzene, *o*-, *m*- and *p*-xylene had similar responses. GRANT[3], using nitrogen as the carrier gas, found that *o*-xylene had a lower response than either *m*- or *p*-xylene which had similar responses.

*Experimental.* Gas–liquid partition chromatography was carried out using the apparatus and liquid phases described previously[1]. Nitrogen was used as the carrier gas at a flow-rate of approximately 33 ml/min, and the bridge current was 100 mA.

It was convenient to use two comparison compounds, benzene for a column temperature of 95°, and *p*-cymene for column temperatures between 125° and 145°.

TABLE I

RELATIVE DETECTOR RESPONSE TO BENZENE HYDROCARBONS AND HALOGEN DERIVATIVES

| *Compound* | *Mol. wt.* | *Response per mole relative to benzene (= 100)* |
|---|---|---|
| Benzene | 78 | 100 |
| Toluene | 92 | 121 |
| Ethylbenzene | 106 | 144 |
| *o*-Xylene | | 128 |
| *m*-Xylene | | 143 |
| *p*-Xylene | | 145 |
| *n*-Propylbenzene | 120 | 162 |
| Isopropylbenzene | | 147 |
| 1,3,5-Trimethylbenzene | | 165 |
| 1,2,4-Trimethylbenzene | | 155 |
| *n*-Butylbenzene | 134 | 193 |
| *sec.*-Butylbenzene | | 183 |
| *tert.*-Butylbenzene | | 174 |
| *p*-Cymene | | 180 |
| 1,2,4,5-Tetramethylbenzene | | 181 |
| 1,2,3,5-Tetramethylbenzene | | 180 |
| | | *Response per mole relative to p-cymene (= 100)* |
| Chlorobenzene | 112.6 | 85 |
| *o*-Chlorotoluene | 126.6 | 95 |
| *m*-Chlorotoluene | | 106 |
| *p*-Chlorotoluene | | 106 |
| Benzyl chloride | | 105 |
| Bromobenzene | 157 | 108 |
| *o*-Bromobenzene | 171 | 113 |
| *m*-Bromobenzene | | 126 |
| *p*-Bromobenzene | | 128 |

Under these conditions no reversal of peaks was observed for any of the compounds studied.

*Results and discussion.* The relative detector responses to a number of monocyclic aromatic hydrocarbons and halogen derivatives are shown in Table 1, and the relative detector responses to a number of phenols and phenol ethers are shown in Table II.

TABLE II

RELATIVE DETECTOR RESPONSE TO PHENOLS AND PHENOL ETHERS

| *Compound* | *Mol. wt.* | *Response per mole relative to p-cymene (= 100)* |
|---|---|---|
| Phenol | 94 | 48 |
| *o*-Cresol | 108 | 59 |
| *m*-Cresol | | 65 |
| *p*-Cresol | | 64 |
| Benzyl alcohol | | 68 |
| 2-Ethylphenol | 122 | 76 |
| 4-Ethylphenol | | 84 |
| 2,3-Dimethylphenol | | 77 |
| 2,4-Dimethylphenol | | 82 |
| 2,5-Dimethylphenol | | 80 |
| 2,6-Dimethylphenol | | 76 |
| 3,4-Dimethylphenol | | 81 |
| 3,5-Dimethylphenol | | 86 |
| 3-Methyl-5-ethylphenol | 136 | 104 |
| 2,3,5-Trimethylphenol | | 93 |
| 2,4,5-Trimethylphenol | | 92 |
| 2,4,6-Trimethylphenol | | 93 |
| *o*-Chlorophenol | 128.6 | 83 |
| *m*-Chlorophenol | | 93 |
| *p*-Chlorophenol | | 93 |
| 4-Chloro-2-methylphenol | 142.6 | 100 |
| 4-Chloro-3-methylphenol | | 101 |
| Anisole | 108 | 66 |
| Phenetole | 122 | 85 |
| *o*-Cresyl methyl ether | | 74 |
| *m*-Cresyl methyl ether | | 83 |
| *p*-Cresyl methyl ether | | 82 |
| *o*-Cresyl ethyl ether | 136 | 90 |
| *m*-Cresyl ethyl ether | | 102 |
| *p*-Cresyl ethyl ether | | 103 |
| *o*-Chloroanisole | 142.6 | 101 |
| *m*-Chloroanisole | | 113 |
| *p*-Chloroanisole | | 113 |

An examination of these results shows that, within a homologous series, there is an increase in relative detector response with an increase in molecular weight. Also, for isomeric compounds, there is a decrease in relative detector response with an increase in chain branching, *e.g.* for the butylbenzenes, the relative detector responses decrease in the order normal, secondary, tertiary.

In all the types of compounds studied it is found that the *ortho*-isomer has a lower relative detector response than either the *meta*- or *para*-isomer, these last two having similar relative detector responses. It is also found that, *ortho*-effects being absent, a compound with a group forming part of a side-chain has a similar relative detector response to the isomeric compound which has the group attached to the benzene ring, *e.g.* *n*-propylbenzene has a relative detector response similar to 1,3,5-trimethylbenzene, but 1,2,4-trimethylbenzene, which has a pair of *ortho*-methyl groups, has a lower relative detector response; also, phenol ethers have similar relative detector responses to the isomeric phenols.

The introduction of a hydroxyl or an alkoxyl group into a benzene ring lowers the relative detector response, since phenols and phenol ethers have much lower relative detector responses than would be expected from benzene hydrocarbons of similar molecular weights.

The introduction of a chlorine atom into the ring of a phenol or a phenol ether has little effect on the relative detector response since the chlorophenols and chlorophenol ethers have relative detector responses similar to those expected from the phenols or phenol ethers with similar molecular weights. Chlorobenzene has a relative detector response similar to that expected from a benzene hydrocarbon of similar molecular weight; *m*- and *p*-chlorotoluenes have relative detector responses slightly higher than the expected values.

*Acknowledgements.* Thanks are due to the Coal Tar Research Association for a pure sample of 1,2,3,5-tetramethylbenzene and to Robert Haldane & Co. Ltd. for very generous samples of the dimethylphenols and the trimethylphenols.

*Chemistry Department, The Paisley Technical College,*
*Paisley (Great Britain)*
G. R. JAMIESON

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## An apparatus for elution from paper chromatograms

There is a long-standing need for an easily-constructed and easily-operated apparatus for eluting substances from paper strips with small volumes of solvents, particularly for those solvents which are so volatile that the whole eluting system must be enclosed. Serial equilibration of the whole or dissected paper with aliquots of the solvent in small vessels does not always meet the need, for the resulting solution is dilute and bulky and must often be reduced in volume before the eluted substances can be further treated. Also, for large pieces of paper it is difficult to get complete contact between paper and solvent in a small vessel. The following simple apparatus is offered for eluting with small volumes of the most volatile solvents from large pieces of paper.

Fig. 1 shows the unmounted assembly. A glass tube of suitable length and diameter to contain the paper to be eluted has a standard-taper socket at the top and a cone at the bottom. The cone that fits the top socket has a closed base and an open top, and a hole is drilled through the wall of the tapered cone at a convenient height above the closed base. (In our apparatus, for the elution of paper strips $9\frac{1}{2} \times 1$ in., the top joint is a B-34, and the bottom one a B-24. The hole is $^1/_8$ in. diameter.) A paper wick of the T-form shown is slotted to one end of the paper strip to be eluted, and the papers placed in the tube in such a way that the T-shaped wick lines the top joint. The cross-bar of the T should project neither above nor below the joint, and the two ends of it should nearly meet around the cone. The cone is fitted into the socket, holding the paper, and with the drilled hole opposite to the down-stroke of the T-wick. The bottom cone of the tube is fitted into any suitable vessel that is to contain the eluate and, for volatile solvents, this vessel should initially contain a few drops of the eluting solvent to help saturate the atmosphere in the tube. The eluting solvent is poured into the recess of the top cone, whose upper end may be closed with a loose-fitting cork. The solvent spreads from the hole all over the wick and soaks down into the paper at a rate determined by the width of the down-stroke of the wick. For water and solvents of high viscosity, the wick should be of thick (Whatman No. 3) paper; for thinner solvents, Whatman No. 1 paper is suitable.

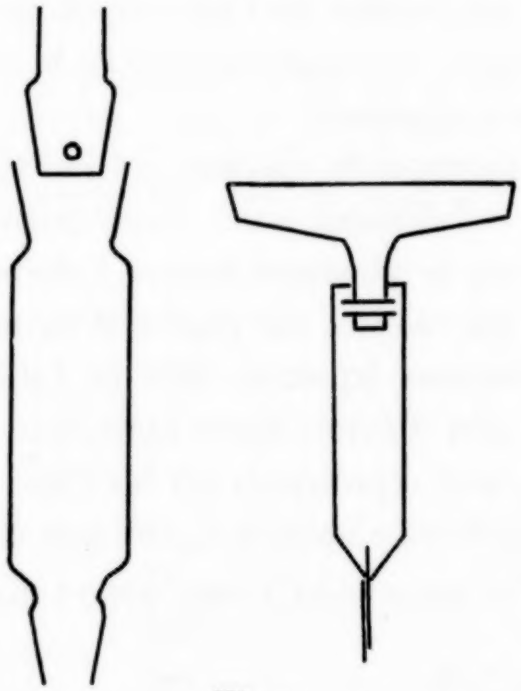

Fig. 1.

The main advantages of this method are: (1) the individual assemblies are cheaply and quickly constructed; (2) the apparatus may be left unattended to complete its elution with the volume of solvent measured into the top reservoir; and (3) large numbers of papers may be eluted simultaneously with a minimum of attention. The apparatus has been found to work equally well with water, acetone, alcohol, ether, etc., and all thicknesses of paper up to Whatman No. 3.

*The Botany School, Cambridge (Great Britain)* M. J. CANNY

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# L'adsorption de quelques chloro-complexes sur cellulose

KRAUS et ses collaborateurs[1] ont récemment montré que plusieurs chloro-complexes anioniques, tels $AuCl_4^-$, $GaCl_4^-$ et $SbCl_6^-$, sont fortement adsorbés par des résines échangeuses de cations (Dowex-50). Ces auteurs ont également noté que cette adsorption augmente avec la concentration de la solution en HCl ou LiCl et que ces anions adsorbables appartiennent tous à la catégorie de ceux qui s'extraient bien par l'éther.

Ces découvertes présentent de nombreuses similitudes avec la chromatographie dite "par relargant" de substances organiques sur résines échangeuses d'ions et dont la description a été faite par RIEMAN et ses collaborateurs[2].

Dans les deux cas, le mécanisme de l'adsorption semble être similaire au processus d'extraction par solvant; le réseau carboné de la résine se comportant comme un solvant (mais en surface seulement).

Nous avons pu noter, au cours de travaux préliminaires sur papier imprégné de Dowex-50, que le papier cellulosique sans imprégnation présente également un pouvoir d'adsorption vis-à-vis de plusieurs anions, chlorés ou bromés.

La chromatographie a été réalisée sur papier Whatman No. 1, selon la méthode ascendante et avec des solutions aqueuses HCl et LiCl; nous avons ajouté à ces dernières 1% de HCl 10 $N$ afin d'éviter toute hydrolyse. Les résultats obtenus avec Au(III), Ga(III) et Fe(III) sont représentés sur les Figs. 1 et 2.

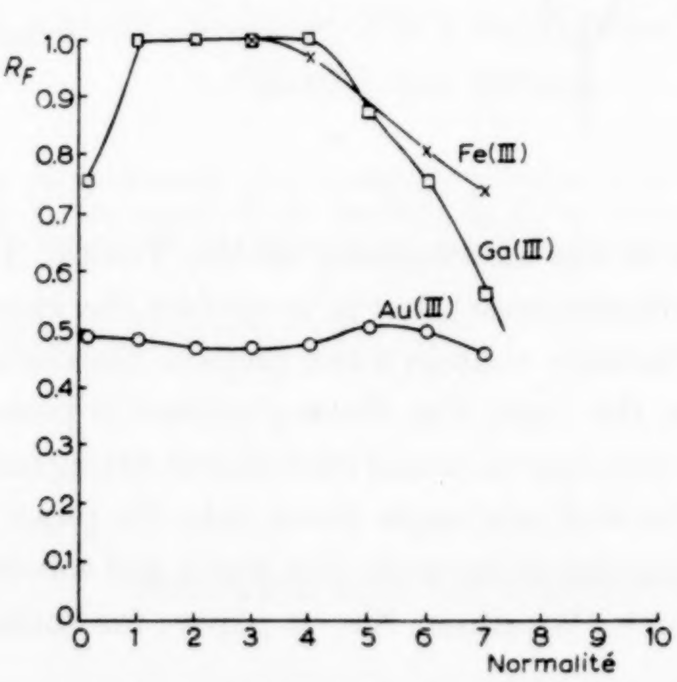

Fig. 1. Valeurs de $R_F$ avec HCl comme solvant.

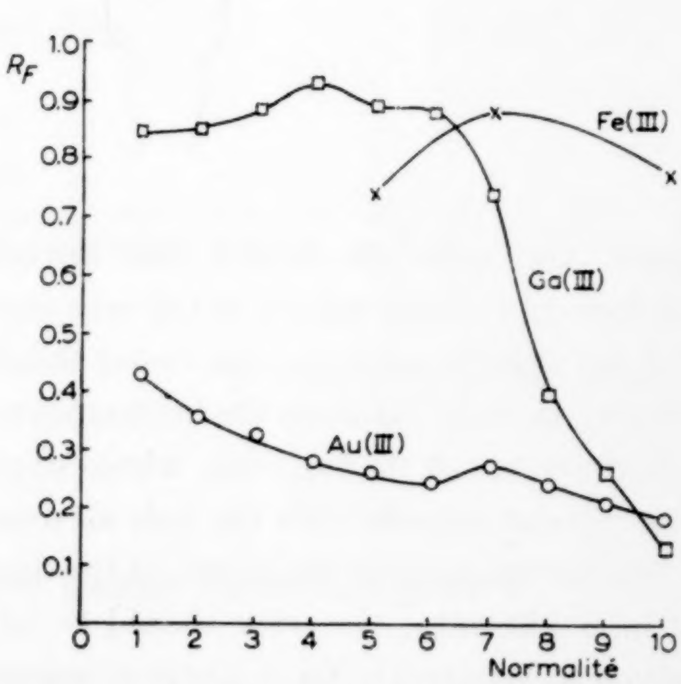

Fig. 2. Valeurs de $R_F$ avec LiCl (contenant 1 % HCl) comme solvant.

Nous avons déterminé les coefficients d'adsorption à l'aide de l'équation $\alpha = (1/R_F - 1)\, A_l/A_s$ en établissant le rapport $A_l/A_s$ par pesées, en boîtes à tare, de carrés de papier avant et après développement.

D'une façon générale, l'adsorption sur cellulose est beaucoup plus faible que sur Dowex-50. C'est ainsi que le coefficient d'adsorption est de 1.5 pour $AuCl_4^-$ en milieu HCl 6 $N$ alors qu'il est de 100 pour la résine Dowex-50. Ces coefficients sont respectivement de 0.55 et 10 pour $GaCl_4^-$ en milieu HCl 6 $N$.

Les résultats offrent des possibilités à l'analyse, en effet les taches obtenues sont assez compactes et le développement est beaucoup plus rapide que dans la plupart des séparations par solvant.

Des travaux portant sur une cinquantaine d'ions susceptibles de former des chloro et des bromo-complexes sont actuellement en cours; pour le moment nous avons déjà noté les coefficients pour Po(IV) et Sb(V) sous différentes conditions. Nous étudions également l'adsorption en fonction de différents facteurs: température, électrolytes et papiers divers.

*Institut du Radium, Laboratoire Curie, Paris (France)*

T. J. BECKMANN
M. LEDERER

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# Zur Technik des Nachweises von Peptiden auf Papier mit Hilfe von Chlor und o-Tolidin

Die von RYDON UND SMITH[1] eingeführte und von verschiedenen Autoren[2–4] weiter entwickelte Methode des Nachweises von Peptiden auf Papierchromatogrammen und Pherogrammen hat vermutlich infolge technischer Schwierigkeiten noch immer nicht die Verbreitung und Anwendung gefunden, die sie eigentlich verdient, da sie vorläufig die einzig auf alle Peptide anwendbare Nachweismethode ist. In unseren seit mehreren Jahren laufenden Untersuchungen an Peptiden und Peptonen[5,6] hat sich folgende Methode bewährt (Fig. 1):

In einer mit einem seitlichen Auslauf am Boden versehenen Wulff'schen Flasche wird Chlorgas dadurch entwickelt, dass auf etwa 30–40 g Kaliumpermanganat rauchende Salzsäure aus einem Tropftrichter zufliesst. Das sich entwickelnde Chlorgas wird aus dem 2. Hals der Flasche über einen Dreiwegehahn, der eine Evakuierung des

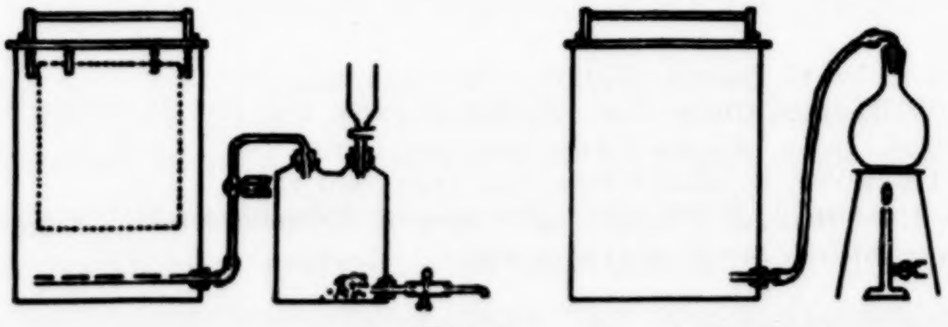

Fig. 1

ganzen Systems mit Hilfe einer Wasserstrahlpumpe erlaubt, in einen hohen, flachen Behälter aus Glas oder emailliertem Blech (wir benutzten dazu Tanks für die Entwicklung von Röntgenfilmen) geleitet. Das Gefäss ist durch einen Holzdeckel, an dem mit 2 Halterungen in etwa 8 cm Abstand ein Glasstab zum Anklammern der Chromatogramme beziehungsweise Pherogramme angebracht ist, verschlossen. Einige Minuten Chlorentwicklung genügen, um den Behälter mit Chlor ausreichend zu füllen. Das an dem nach oben gedrehten Glasstab mit Klammern befestigte Papier wird durch Besprühen mit einer Mischung aus Aceton und 30 %igem Alkohol zu gleichen Teilen mässig angefeuchtet, der Deckel um seine Längsachse gedreht und das Papier in das Chlor versenkt. Nach einer Reaktionszeit von 3–10 Minuten wird der Deckel wieder nach oben gedreht, das Chromatogramm nunmehr auf einen gleichartigen Deckel eines weiteren Gefässes, das durch leichtes Erwärmen einer konzentrierten Ammoniaklösung in einem Kolben mit durchbohrtem Gummistopfen und Schlauchverbindung mit $NH_3$ gefüllt wurde, geklammert, und für 5–10 Sekunden nach dem Vorschlag von[3] zur Erzielung eines hellen Untergrundes der Einwirkung von Ammoniak ausgesetzt. Es muss unbedingt eine zu lange Behandlung mit Ammoniak vermieden werden, man prüft durch Besprühen einer Ecke des Papiers mit dem Tolidin-Reagens, ob noch eine Blaufärbung des Untergrundes vorhanden ist und behandelt gegebenenfalls erneut für 5–10 Sekunden mit Ammoniak. Im Gegensatz zum vielfach geübten Durchziehen des Papiers durch das Tolidin-Reagens nach der Chlorierung wird nach unserem Verfahren mit dem Reagens (gesättigtes *o*-Tolidin in 2 %iger Essigsäure mit gleichem Teil an $M/20$ Kaliumjodid-Lösung) besprüht. Die blau-schwarzen, bei hohen Konzentrationen auch braun-gelben Flecke der umgesetzten Aminosäuren oder Peptide erscheinen sofort und bleiben für kürzere oder längere Zeit je nach Konzentration und Art des geprüften Stoffes bestehen. Das Besprühen vermeidet die Möglichkeit des Auswaschens leicht löslicher Stoffe beim Durchziehen durch das Tolidin-Reagens. Die Flecke können durch Photographie oder Photokopie dokumentiert werden.

Ein Vorteil des Verfahrens besteht darin, dass die Reste der Reaktion nach Öffnen der Sperrklammer durch Spülen mit reichlich Wasser leicht entfernt werden können. Es ist zweckmässig, alle Massnahmen unterm Abzug auszuführen.

*Physiologisch-Chemisches Universitäts-Institut,* O. J. SCHMID
*Hamburg* (*Deutschland*)

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Eingegange [illegible]. Januar 1960

# Ein einfaches Gerät zur Auftragung der Untersuchungssubstanz bei der kontinuierlichen Papierelektrophorese

Bei analytischen oder präparativen Arbeiten mit Hilfe der kontinuierlichen Papierelektrophorese erfolgt die Auftragung der Untersuchungssubstanz üblicherweise mittels eines Dochtsystems oder einer recht kostspieligen Dosierpumpe. Bei dieser wird durch einen elektrischen Synchronmotor der Kolben einer Fortunapipette oder einer Injektionsspritze gleichmässig bewegt; die Einstellung der Zuführungsgeschwindigkeit geschieht durch ein Übersetzungsgetriebe und durch Verwendung von Spritzen mit unterschiedlichen Querschnitten.

Anstelle dieses Verfahrens verwenden wir seit mehreren Jahren eine wesentlich einfachere Anordnung, die sich ausgezeichnet bewährt hat; sie beruht auf dem Prinzip des Knallgasvoltameters und lässt sich mit geringem Aufwand leicht herstellen.

Die Apparatur besteht, wie aus Fig. 1 ersichtlich, aus einem Elektrolysekölbchen F von 15 mm ø mit 2 eingeschmolzenen Pt-Elektroden, einem 2-Wegehahn C mit kapillaren Schenkeln, der durch Schliffe (NS 7.5) mit dem Kölbchen auf der einen Seite und einer 2-ml-Pipette B auf der anderen Seite in Verbindung steht. Die Spitze der Pipette läuft über einen kleinen Schlauch in eine Kapillare A aus, die zur Auftragung der Substanz an das Elektrophoresepapier angelegt wird. Über die Kapillare ist ein durchbohrter Gummistopfen gestreift, der zur Befestigung der Kapillare an der Glaswand der Elektrophoreseapparatur dient; das Elektrolysekölbchen wird durch eine allseitig schwenkbare Halterungsvorrichtung an der Elektrophoreseapparatur angebracht.

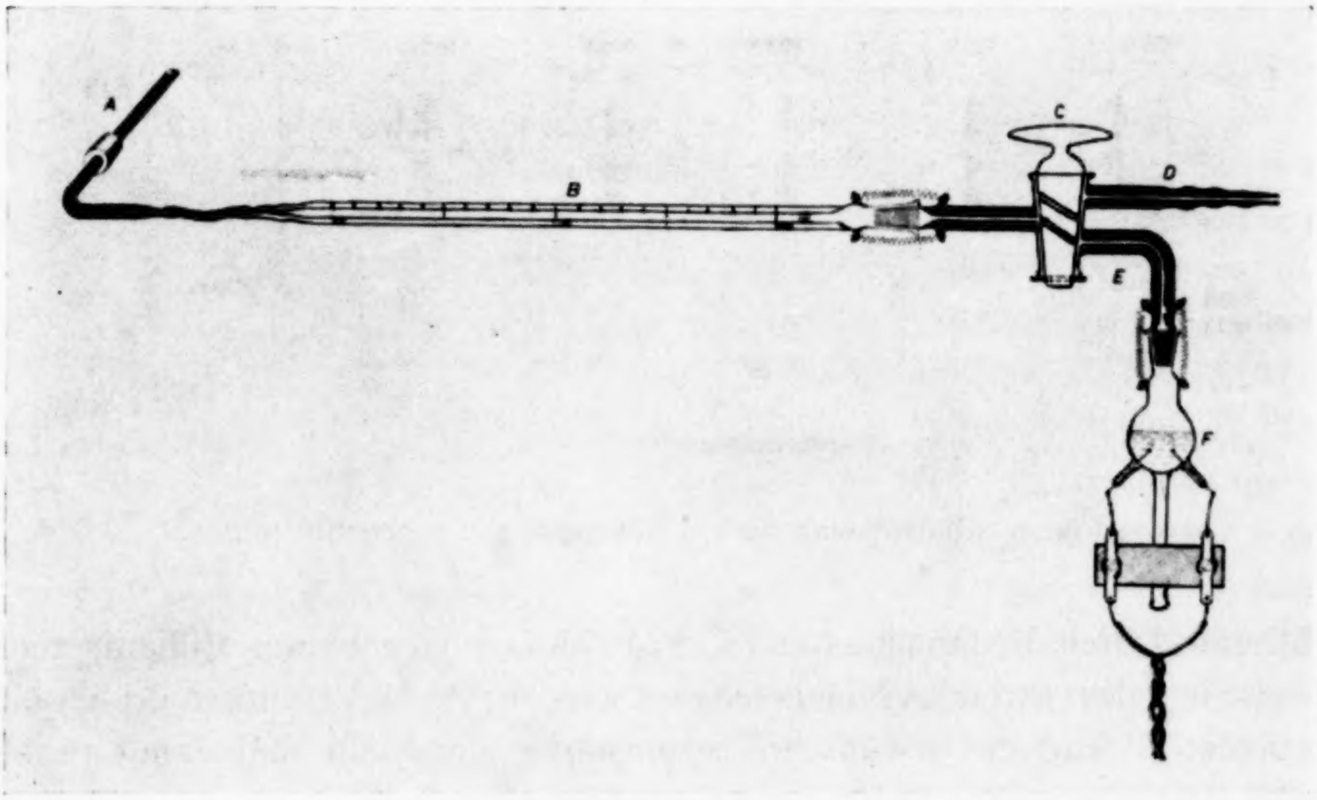

Fig. 1. Auftragungsgerät für die kontinuierliche Papierelektrophorese.

Zur Auftragung wird die Untersuchungssubstanz bei entsprechender Stellung des

2-Wegehahnes durch Saugen an dem Kapillarschenkel D durch die Kapillare A bis zur 0-Marke in die Pipette eingesaugt. Anschliessend wird die Kapillare A auf das Papier angesetzt, der 2-Wegehahn auf den Schenkel E umgestellt und durch Stromzuführung in dem Elektrolysekölbchen, das etwa zur Hälfte mit 0.0005 $N$ $H_2SO_4$ gefüllt ist, Knallgas erzeugt. Durch die Gasentwicklung wird die Untersuchungssubstanz langsam und gleichmässig aus der Pipette herausgedrückt und dem Papier appliziert.

Als Stromquelle dient eine 15-V-Trockenbatterie, bei der mit Hilfe eines Potentiometers (250 $\Omega$) in einfacher Weise die Spannung und damit die Stromstärke geregelt werden kann, oder bei Verwendung mehrerer Apparaturen ein einfaches Netzgerät; Fig. 2 zeigt das Schaltschema eines solchen Wechselstromgerätes mit 5 verschiedenen Anschlüssen. Durch die Drucktasten ($S_6$–$S_{10}$) kann ein eingebautes Milliamperemeter wahlweise in jedem Stromkreis eingeschaltet werden; durch Verändern des jeweiligen Potentiometers wird die gewünschte Stromstärke eingestellt und damit praktisch stufenlos jede gewünschte Auftragungsgeschwindigkeit.

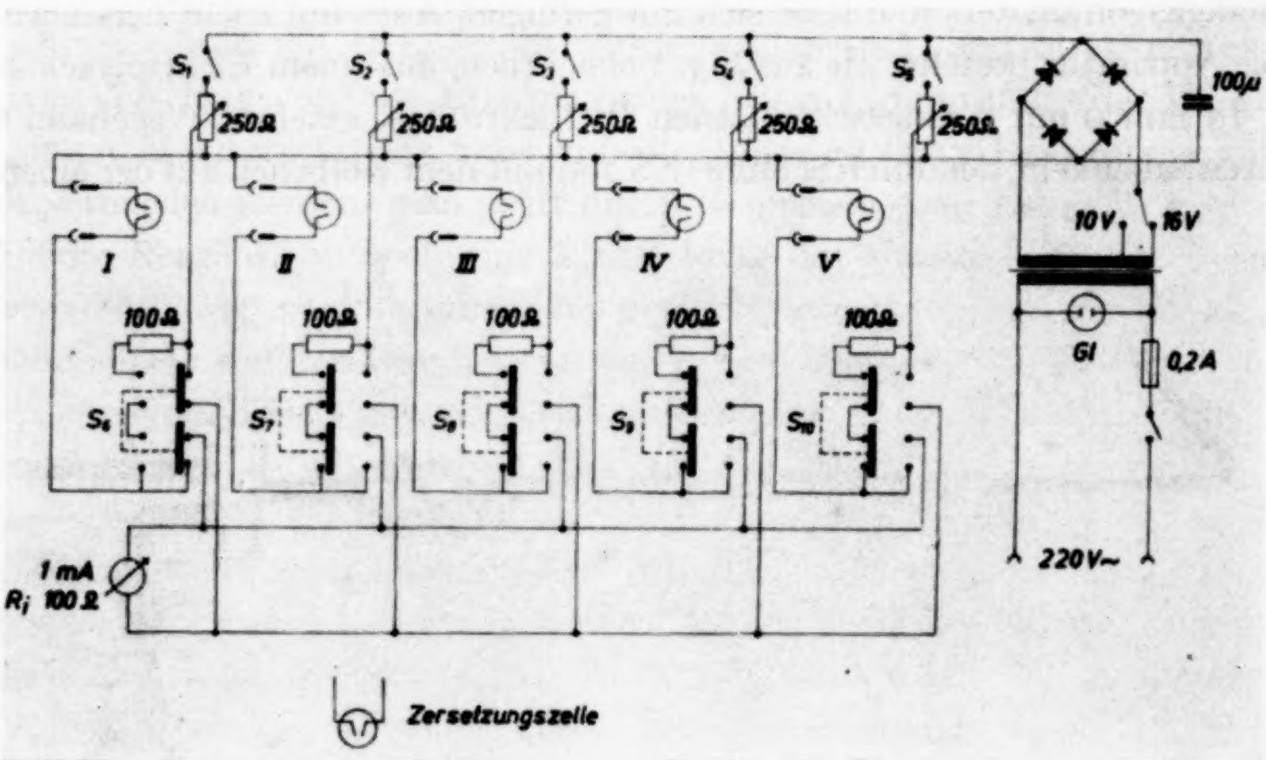

Fig. 2. Schaltschema für ein Netzgerät mit 5 Anschlüssen.

In einer Stunde erzeugen 0.1 mA 0.062 ml Knallgas; um in einer Stunde z.B. 0.1 ml Untersuchungssubstanz applizieren zu können, werden ca. 0.16 mA benötigt, d.h. bei einem Innenwiderstand des Elektrolysekölbchens von etwa 30 k$\Omega$ muss eine Spannung von etwa 5 V angelegt werden.

*Institut für Pflanzenzüchtung der Deutschen Akademie der Landwirtschaftswissenschaften, Quedlinburg (Deutschland)*

WERNER MATTHIAS

Eingegangen den 19. Januar 1960

# A modified method for the paper chromatography of long-chain fatty acids

Although quantitative analysis of fatty acids is best performed by vapour-phase chromatography after conversion to the methyl esters, nevertheless paper chromatography offers certain advantages for identification and semi-quantitative estimation. Thus the preparation of samples for analysis is simplified, since the free fatty acids can be separated on paper without prior esterification; and of course, the simplicity of both apparatus and technique is an attractive feature.

Numerous methods have been described[1–4] for separating long-chain fatty acids on paper, involving reversed-phase chromatography. Similarly, many spray reagents have been developed for visualising the spots. Of these, quantitative reactions usually depend on forming a metal soap with subsequent development of a coloured complex with the metal. For qualitative and semi-quantitative work, an indicator, such as bromphenol blue is frequently employed.

The purpose of the present communication is to describe a simplified version of previous separation procedures and to suggest the use of a different spray solution that gives a clear demonstration of spots even with very small quantities of acids.

For the preparation of the papers, Whatman No. 3 paper, in strips of 23 × 5 cm, were immersed for about 5 min in a 10% (v/v) solution of liquid paraffin (B.P.) in ether, contained in a flat dish. It was considered advisable to cover the dish so as to avoid undue evaporation of ether but this point was not critical; neither was the time of impregnation, provided that at least 5 min soaking was allowed. At the end of this period, the impregnated paper strips were hung in the fume-cupboard to allow the ether to evaporate. At this stage, the papers are ready for chromatography, though it may be preferable to leave them overnight before use. Similarly, although the impregnated papers remain efficient on storing for a few days, it has been observed occasionally that storage for longer than two weeks results in poor separation of acids.

The solvent system used was a 4:1 solution (v/v) of acetone–water. It has not been found necessary to equilibrate solvent with stationary phase (liquid paraffin), but the purity of the acetone is an important factor. Analytical grade acetone may be used without redistillation, but commercial quality solvent is unsuitable.

The separation of long-chain saturated fatty acids has been achieved with the reversed-phase papers and solvent described by means of ascending chromatography. Equilibration of papers with solvent vapour for lengthy periods is unnecessary, although it may be advantageous to allow a short period (30 min). The time required for good separation is about 3 h, involving movement of the solvent front of approximately 12 cm from the starting point. In this procedure, typical $R_F$ values are as follows:

| | |
|---|---|
| Stearic acid | 0.23 |
| Palmitic acid | 0.40 |
| Myristic acid | 0.55 |

With such convenient values of $R_F$, lower acids down to $C_{10}$, and slightly higher acids can be detected on the same chromatogram. If a larger range of chain length is required, impregnation of the paper with a lower concentration of paraffin allows the acids to travel faster, and the same effect can be achieved by increasing the acetone content of the solvent.

The spray reagent is essentially an alkaline solution of Nile Blue, so that the background colour is pale red, whilst the fatty acids show up as blue spots. The reagent is prepared as follows:

Nile Blue sulphate solution (0.2 % in ethanol) 50 ml
Triethanolamine 15 ml (approximately)
Ethanol 50 ml

Triethanolamine is added to the stock solution of Nile Blue sulphate in ethanol until the blue colour changes to a clear red, untinged by blue. The solution is then diluted with ethanol.

At first ammonia was tried as the means for providing an alkaline medium, but this is volatile, and the background of the sprayed chromatogram quickly turned blue owing to $CO_2$ in the atmosphere. Triethanolamine is advantageous in that it remains on the paper, and the colour change of the background is therefore retarded.

In order to achieve the utmost simplicity of technique, no precautions have been taken in this method of separation to eliminate variables; and hence $R_F$ values tend to change, most frequently because of change in temperature. Because of such considerations, it is always essential to run a control sample, preferably a known mixture of myristic, palmitic and stearic acids. Another fault, inherent in all similar techniques, is that unsaturated acids give the same $R_F$ value as the saturated acid containing two carbon atoms less.

Nevertheless, good separations are obtained by this method, and the chromatogram can be sprayed immediately after development, without the need for lengthy treatment as in some other techniques. The method has been used successfully for the preliminary examination of fatty acids from biological tissues.

*Acknowledgements*

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*Department of Chemical Engineering, University College,*
*London (Great Britain)*

R. Chayen
E. Margery Linday

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Received February 1st, 1960

# Positive identification of flavanone aglycones by paper chromatography of their alkaline degradation products

Studies of flavanones found in citrus fruits revealed the need for an improved method of identifying very small quantities of closely related flavanone aglycones, such as hesperetin, homoeriodictyol, and isosakuranetin, which are difficult to identify unequivocally by paper chromatography because of very similar $R_F$ values and color reactions. Such a method has, therefore, been developed in our laboratory. In this method, paper chromatography is utilized for identification of the degradation products (phloroglucinol and substituted cinnamic acids) which are obtained by adapting to semi-micro scale the classical aqueous potassium hydroxide degradation of flavanone aglycones. Chromatographic data for phloroglucinol and several substituted cinnamic acids which are, or might be, produced on degradation of flavanone aglycones are presented in Table I. The data show that these compounds can be differentiated and positively identified on the basis of their $R_F$ values in different solvent systems, of their fluorescence under ultraviolet light in air and when exposed to ammonia vapor and of their color reactions with chromogenic spray reagents. Hence, if a small quantity of a flavanone aglycone is properly degraded, the degradation products can be positively identified by paper chromatography, and the identity of the original molecule can thus be established with certainty.

Degradation of flavanones may be satisfactorily accomplished by mixing the aglycone with a small quantity of 30% aqueous potassium hydroxide (usually about 2–3 ml of alkali per mg of aglycone) and refluxing for 2.5 h. The degradation mixture is then made acidic (pH 4) with dilute sulfuric acid, and extracted 3 times with ethyl ether. The ether extract is washed twice with small quantities of water, reduced in volume and chromatographed in comparison with standard substituted cinnamic acids and phloroglucinol.

FEWSTER AND HALL'S[1] *n*-butyl alcohol: ethyl alcohol: ammonia–ammonium carbonate buffer (40:11:19 v/v/v) solvent system, and a modification of this system prepared by mixing 1 part nitromethane with 2 parts of *n*-butyl alcohol saturated with ammonia–ammonium carbonate buffer (1.5 *N* with respect to both ammonia and ammonium carbonate) are very useful solvent systems for identification of the cinnamic acids produced on degradation. These systems give small, concentrated spots with no tailing, and therefore permit easier location of weak fluorescing spots and give more clearly defined spots with indicator spray reagents, particularly for very low concentrations of acids. They are especially suitable for chromatography of cinnamic acids containing only methoxy substituents. Table I indicates that observation of $R_F$ values in these solvents, plus fluorescence, permits differentiation between such closely related compounds as *o*-, *m*-, and *p*-methoxycinnamic acids.

Diazotized sulfanilic acid[2] and *p*-nitroaniline[3] spray reagents are profitably employed with chromatograms of the cinnamic acids containing hydroxyl groups, and phloroglucinol, not only to assist in locating the spots, but also to produce colors which are often characteristic and useful in identification. For chromatograms of the

TABLE I

$R_F$ VALUES, FLUORESCENCE AND COLOR REACTIONS OF SUBSTITUTED CINNAMIC ACIDS AND PHLOROGLUCINOL

| *Compound* | *$R_F$ values in given solvent*[a] | | | | *Fluorescence and color reactions with reagent given* | | | |
|---|---|---|---|---|---|---|---|---|
| | *n-Butyl alcohol–acetic acid–water (6:1:2)* | *n-Butyl alcohol: ethyl alcohol: $NH_3$–$(NH_4)_2CO_3$ buffer* | *n-Butyl alcohol: nitromethane: $NH_3$–$(NH_4)_2CO_3$ buffer* | *Chloroform–acetic acid–water (2:1:1)* | *Ultraviolet light* | *Ultraviolet light in $NH_3$ fumes* | *Diazotized sulfanilic acid spray reagent* | *Diazotized p-nitroaniline spray reagent* |
| *o*-Coumaric acid | 0.88 | 0.47 | 0.34 | 0.85 | blue-white | yellow-green | orange | wine |
| *p*-Coumaric acid | 0.86 | 0.44 | 0.26 | 0.79 | negative | bright blue | red-pink | gray-purple |
| Ferulic acid | 0.81 | 0.31 | 0.25 | 0.92 | blue | sky blue | purple | gray-purple |
| Isoferulic acid | 0.83 | 0.40 | 0.29 | 0.90 | blue | tan or cream | orange-pink | dark purple |
| Phloroglucinol | 0.70 | 0.79 | c | c | negative | blue | yellow-brown | brown |
| *o*-Methoxycinnamic acid | b | 0.61 | 0.38 | d | blue | fades | e | e |
| *m*-Methoxycinnamic acid | b | 0.59 | 0.34 | d | blue | fades | e | e |
| *p*-Methoxycinnamic acid | b | 0.54 | 0.27 | d | deep purple (faint) | fades | e | e |
| 3,4 Dimethoxycinnamic acid | b | 0.44 | 0.28 | d | blue | fades | e | e |

a. Whatman No. 1 paper. Ascending chromatography with all solvent systems except *n*-butyl alcohol–acetic acid–water.
b. Spots produced are too diffuse for accurate location by fluorescence or reaction with indicator spray reagents.
c. Compound streaks or tails badly in these solvents.
d. Compounds move with solvent front.
e. Compounds do not couple with these diazotized reagents.

cinnamic acids possessing only methoxy substituents, and which, therefore, will not couple with the diazotized reagents mentioned, indicator solutions such as buffered methyl red (0.1% alcoholic methyl red–0.167 $M$ phosphate buffer, 1:2) and 2,6-dichlorophenol-indophenol (0.4% in 95% ethyl alcohol) are useful as spray reagents for locating the acid spots, particularly those with weak fluorescence.

Employing the procedures outlined here, we have successfully degraded 1 mg samples of the flavanone aglycones hesperetin, homoeriodictyol, isosakuranetin, and naringenin, and identified their degradation products by paper chromatography. We have not obtained satisfactory results with eriodictyol, apparently because the caffeic acid which should be produced on degradation of this compound is destroyed by the alkaline degradation conditions. This method has been employed successfully in our laboratory for positive identification of very small quantities of unknown flavanone aglycones obtained by hydrolysis of glycosides from citrus fruits.

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*Chemistry Department, University of Oklahoma,*
*Norman, Okla. (U.S.A.)*

WILLIAM J. DUNLAP
SIMON H. WENDER

1 M. E. FEWSTER AND D. A. HALL, *Nature*, 168 (1951) 78.
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## BOOK REVIEW

*Chromatographie en chimie organique et biologique*, Vol. I, edited by E. LEDERER (Collection de monographies de chimie organique, compléments au *Traité de Chimie organique*), Masson et Cie, Paris, 1959, 671 pages.

This is the first book on chromatography to be written in French since 1949. Volume I is divided into two parts: *Généralités* (358 pages) and *Applications de la chromatographie en chimie organique* (276 pages), while Volume II will deal with the applications of chromatography to biochemistry.

Part I consists of an excellent account by CHOVIN of the theory and methods of adsorption chromatography (110 pages), which is well illustrated, and gives an adequate and clear account of the theory. The chapter on ion exchange (by BUC, 34 pages) is rather superficial and in places not quite clear. A table on the properties of resins, for example (page 120), has a column entitled "temperature" without specifying that the maximum operating temperature is meant. It could also be the optimum temperature.

An excellent treatise on partition chromatography was written by BOULANGER AND BISERTE (92 pages), who give a wealth of practical information, for example on desalting, Fig. 23. CHOVIN AND LEBBE deal in the following chapter with the principles of gas chromatography. This seems to be the first account of this method in French. This chapter alone will make the book invaluable for all French-speaking chemists. The last chapter of Part I, by ROCHE, LISSITZKY AND MICHEL, is a thorough account of radioactivity techniques.

Part II starts with a chapter by BESTOUGEFF on hydrocarbons, written rather from the point of view of an analytical chemist and containing only three tables of retention volumes and $R_F$ values.

The chapter on alcohols by DEMOLE gives a detailed account with numerous tables of $R_F$ values. Occasional small paragraphs mention some important electrophoretic methods. Phenols are dealt with by DIETRICH who mentions solvent systems and reagents (in detail) of most of the useful methods. It is a pity that he did not include the work published in the last two years. Aldehydes and ketones are treated in the same manner as alcohols by DEMOLE. A rather long chapter (divided into lower and higher fatty acids and aromatic acids) by ASSELINEAU gives a good account of the separation of acids. It is one of the merits of the book that it deals with all forms of chromatography unlike similar recent treatises on paper chromatography. Thus the reader can decide which method is most suitable for his purposes.

A short chapter on nitro compounds (by DIETRICH) as well as another on halogen compounds (by E. LEDERER) is of necessity incomplete because most of the compounds of these classes were already mentioned in other chapters. Amines are well discussed by DIETRICH and alkaloids by JANOT AND LE HIR. These present of course only a survey of the principles. STAMM AND ZOLLINGER present the separation of dyestuffs, including much of their own work in this field. The final chapter by JACQUES AND KAGAN deals with stereoisomers.

Both subject and author indexes are well prepared. Unfortunately the volume is not entirely free from printer's errors.

## NEW BOOKS

*Proceedings of the International Symposium on Microchemistry*, held at Birmingham University, August 1958. Published by Pergamon Press, London, 1960, xxvi + 583 pages.

The volume contains all papers presented at the symposium.

*Protides of the Biological Fluids* (Proceedings of the Seventh Colloquium, Bruges, 1959), edited by H. PEETERS, published by Elsevier Publishing Co., Amsterdam, 1960, x + 420 pages, price 76 s.

*Chromatographic and Electrophoretic Techniques*, edited by I. SMITH, published by Wm. Heinemann Medical Books Ltd., London, 1960. Vol. 1, *Chromatography*, 648 pages, 160 illus., price 65 s; Vol. 2, *Electrophoresis*, 236 pages, 100 illus., price 30 s.

Vol. 1 is a revised and enlarged edition of "*Chromatographic Techniques*".

REVIEW

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# STARCH ELECTROPHORESIS

## III. STARCH GEL ELECTROPHORESIS

H. BLOEMENDAL

*Department of Biochemistry, Antoni van Leeuwenhoek-Huis:*
*The Netherlands Cancer Institute, Amsterdam (The Netherlands)*

(Received January 12th, 1960)

A promising electrophoretic method was introduced by SMITHIES[5,6], who applied starch gel as supporting medium*. The idea of using gel electrophoresis was not new in itself; successful experiments with agar gels had been carried out previously by GORDON *et al.*[90,91]. The same supporting medium has already been applied as long ago as 1923 for the separation of inorganic ions[92].

Starch, as compared with other gels, has certain advantages owing to its uncommonly high resolving power. However, the reason why, for instance, serum splits up into more bands than observed in other stabilizing materials is not yet wholly clear. There is a possibility that some of the extra zones are found as a result of interaction between the borate buffer used and certain proteins linked to carbohydrate. Furthermore, a molecular sieving effect may occur. Small molecules will penetrate the swollen gel grains more easily than molecules of larger size. The same principle forms the basis of a new technique called "gel filtration" which may be successfully applied for desalting of protein solutions and separation of these solutions from peptides and amino acids[93]. In this way BOSCH AND BLOEMENDAL[94] were able to free ribonucleic acid components from sodium chloride after fractionation with salt gradients on Ecteola columns.

Whereas a growing stream of papers is being published on starch gel electrophoresis as an analytical tool, no convenient technique for large scale preparative work has so far been described. The paper by BERNFELD AND NISSELBAUM[95] deals with the separation of larger quantities of protein, but these workers used a gel that was not prepared from hydrolysed starch only.

## METHODS

### 1. *Apparatus*

Starch gel electrophoresis may be carried out in similar rectangular plastic boxes as described in the first part of this review[63]. A suitable tray originally used by SMITHIES[6] had the following dimensions: depth, 6.5 mm; width, 20 mm; length, 250 mm. In

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[*] *Note added in proof.* When this review was in print a detailed bibliography of the literature with special reference to studies of serum proteins was published by SMITHIES[151].

starch block electrophoresis a vertical position of the box appeared to be preferable[8,63]; according to a recent paper by SMITHIES[96] a vertical arrangement of the tray also gives better results in the separation of serum components in starch gel electrophoresis. Fig. 12 shows this improved apparatus which is also used in our laboratory.

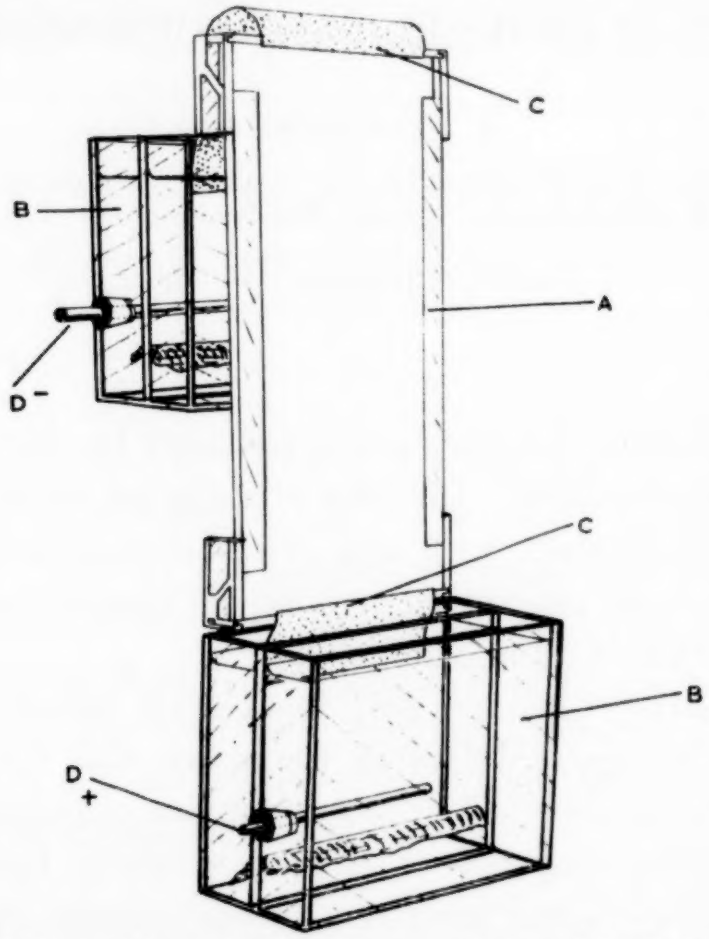

Fig. 12. Vertical starch gel electrophoresis. A. Tray containing the gel. B. Buffer vessels. C. Filter paper wicks. D. Carbon electrodes.

### 2. *Electrodes*

The construction of simple plastic electrode vessels is described in Part I[63]. Platinum, carbon or reversible silver–silver chloride electrodes may be used. The reversible electrodes are easily prepared by electrolysis of silver wire in 0.5 $M$ HCl. The electrode is immersed in a tray containing 10% sodium chloride solution. Filter paper or agar bridges are used to connect the electrode compartment with a vessel containing buffer solution (in serum fractionation 0.06 $M$ NaOH + 0.3 $M$ $H_3BO_3$ is mostly used).

### 3. *Preparation of the gel*

The essential point in the procedure of starch gel electrophoresis is the preparation of a good gel, made from soluble starch. Starch from different batches does not always possess the same gelling properties. Hydrolysed starch ready for use is now commercially available (*e.g.*, Connaught Medical Research Laboratories, University of Toronto, Canada).

Good gels are obtained in the following way:

(a) Pure potato starch is washed with distilled water and borate buffer (or any other buffer solution to be used in the electrophoretic experiment), followed by rinsing with distilled water and dehydration with acetone.

(b) 250 g of the dry starch is suspended in 495 ml acetone containing 5 ml concentrated HCl. This mixture has to stand for 1 h at a temperature of 37°.

(c) A solution of sodium acetate or carbonate is added until the pH is 7. The starch is then resuspended in distilled water and washed thoroughly on a Buchner funnel in order to remove sodium chloride and acetate ions.

(d) The starch is dried by dehydration at 40°.

(e) The hydrolysed starch is mixed with buffer (10–15:100 w/v) with constant swirling. When a temperature of approximately 90° has been reached, the flask is de-aerated under slightly reduced pressure during a few seconds.

(f) The hot gel is poured into the tray and immediately covered with a flexible sheet of plastic, the surface of which is coated with a thin layer of paraffin oil.

(g) The excess of gel is forced out by carefully pressing on the sheet so that air bubbles are avoided. After 2–3 hours cooling in a refrigerator or in a cold room the plastic sheet is removed and the gel is covered with a perspex plate or with a layer of paraffin wax. In the latter case the melted paraffin is poured on the gel just short of congealing (temperature about 45°).

The starch may also be mixed with Hyflo-Super Cel and amylose[95], in this way a stiff gel is obtained in which the electroosmotic flow is extraordinarily low. This gel is easily cut after the electrophoretic run and preparative work becomes possible.

The gel is connected with the outer compartments of the electrode vessels by means of filter paper strips enclosed in parafilm or thin plastic sheets.

Gels may be stored overnight in a cold room or in a refrigerator.

### 4. *Insertion of the sample*

There are three different methods of introducing the sample.

(a) A slit is cut transversely in the gel and filled with the solution to be separated. SMITHIES' improved apparatus has a device[96] which allows the formation of a slit when the gel is poured.

(b) The sample is soaked into a small strip of filter paper. An opening is cut in the gel and the wet paper is put in by means of two forceps. When only small amounts of the sample are available, this method is preferable.

A similar insertion technique is applied when starch gel electrophoresis is carried out in combination with paper electrophoresis[97]. After the sample has been separated on filter paper, the strip is inserted into a slit in the starch gel and the second run is carried out at right angles to the first.

(c) The sample is mixed with starch powder so that a homogeneous paste is obtained. This paste is carefully put into a slit cut in the gel. When the electrophoresis is carried out vertically, melted paraffin wax is poured on the solution so that the slit filled with the sample is sealed. Then the whole gel surface is covered with paraffin wax, after which the tray is brought into a vertical position.

### 5. *Electrophoresis*

In serum analysis the starch gel electrophoresis is mostly carried out as described originally by SMITHIES[5,6,96] (see also Fig. 13).

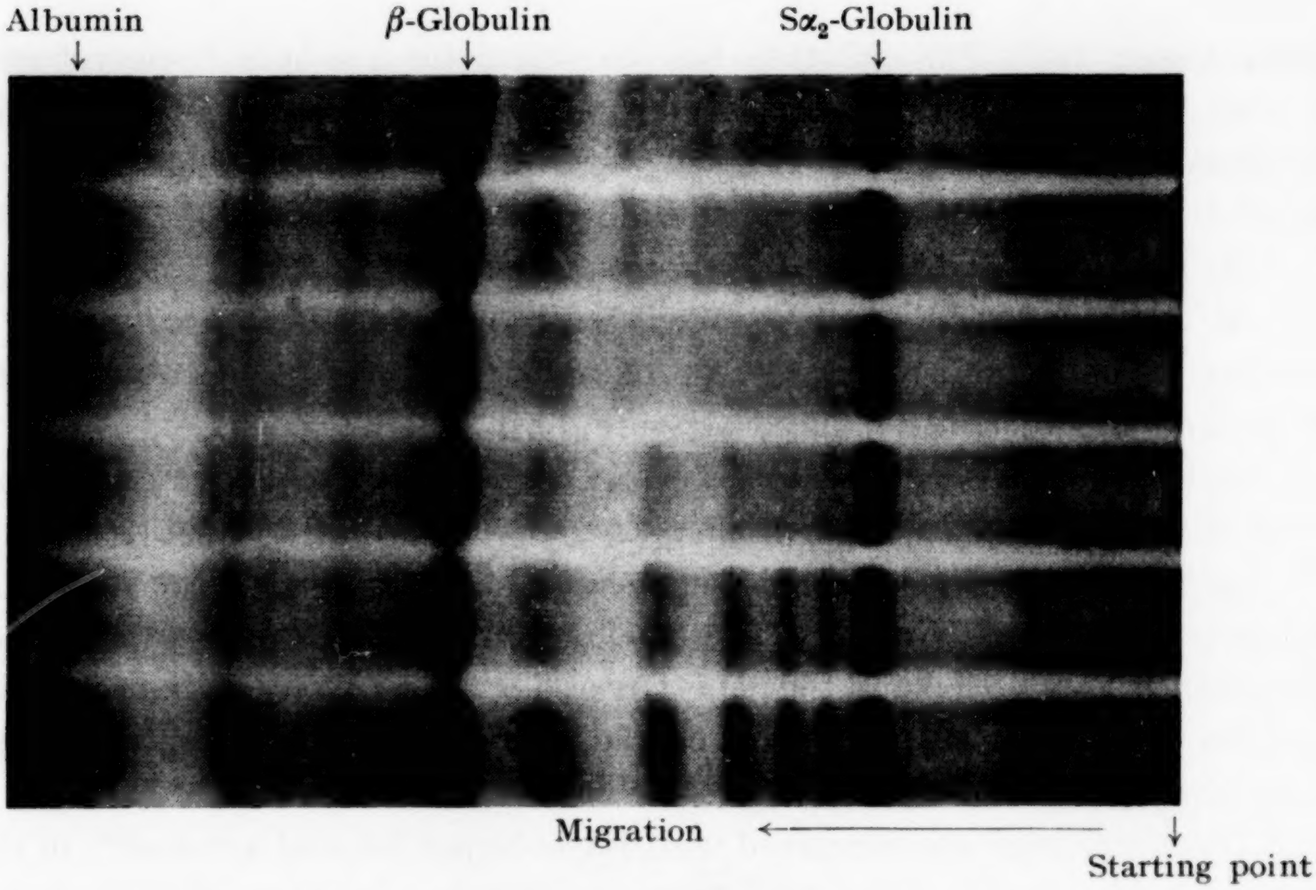

Fig. 13. Serum separation obtained with vertical starch gel electrophoresis (reprinted with the kind permission of Dr. O. SMITHIES and the Editor of *The Biochemical Journal*[96]).

POULIK[98] pointed out that a discontinuous buffer system may have considerable advantages for the separation of certain proteins.

In Table V the working conditions for the fractionation of several substances are given.

TABLE V

| *Material* | *Buffer* | *Ionic strength, molarity* | *pH* | *V/cm* | *mA* | *h* | *Reference* |
|---|---|---|---|---|---|---|---|
| Histone | Unbuffered NaCl solution (adjusted with HCl) | 0.02 $\mu$ | 4.1–4.9 | 3.2 | 3.5 | 6 | 101 |
| Haemocyanin | Borate | 0.02 $M$ + 0.008 $M$ NaOH | 8.03 | 9 | — | 12 | 104 |
| Haemoglobin | Borate | 0.03 $M$ | 8.5 | 6 | — | 5 | 112 |
| Human plasma | Borate | 0.025 $M$ | — | 450 V | — | 18 | 124 |
| Parotid protein | Borate | 0.03 $M$ | 8.4 | 8.7 | 4.5 | 5 | 138 |
| Azotobacter protein | Veronal | 0.1 $\mu$ | 8.6 | 540 V | 50 | 20 | 139 |
| Gastricsin | Acetate | 0.1 $\mu$ | 5 | 4.55 | — | 22 | 144 |
| Thyrotropin | Acetate | 0.012 $\mu$ | 5 | 260 V | 11 | 3–4 | 145 |
| Bromocresol green | Borate | 0.05 $M$ + 0.02 $M$ NaOH | 8.5 | 5 | 1.4 | 5.5 | 146 |

### 6. *Influence of temperature*

Not much is known exactly about the effect of the rise of temperature on starch gel electrophoresis. Like in other stabilizing media and in free electrophoresis the mobilities are temperature-dependent. Heat development is stronger in the strips than on the surface so that distorted zones may be the result of irregularity of the temperature distribution. Using low ionic strength buffers and small strips the temperature effect is reduced, provided the voltage gradient is not higher than 5–6 V/cm.According to GOLDBERG[99] the temperature at which the separation of serum haemoglobin is performed is not critical over a range of 10–25°. At 30°, however, the wicks forming the connection between gel and electrode vessel tend to dry out.

### 7. *Location of substance*

Different procedures are recommended for staining the gels. After the electrophoretic run the gel is carefully taken from the tray. Trays with removable side walls are very convenient. The gel is cut parallel to the bottom surface of the tray so that two equal strips are obtained. A razor blade or microtome may be used to split the gel in a single movement. In our laboratory a very simple apparatus has been constructed to divide the gels (Fig. 14). A thin stainless steel thread is stretched over a slide which fits the bottom of the electrophoretic tray.

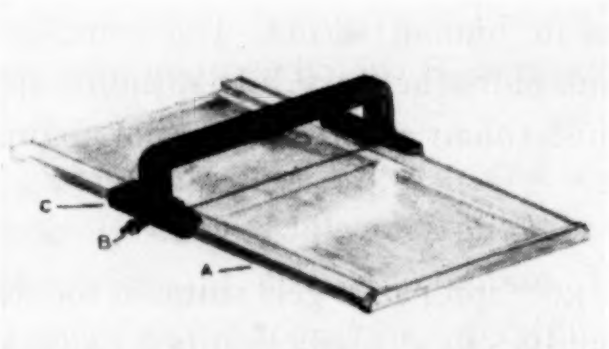

Fig. 14. Slicing device for starch gel electrophoresis. A. Bottom of electrophoresis tray. B. Screw for stretching the thread. C. Metal slide.

Staining is carried out by pouring dye on the cut surface. Many workers employ protein staining with a saturated solution of amido black 10B in methanol–water–glacial acetic acid (50:50:10) during 10–30 min. Excess of dye is removed by 3–4 washings with the solvent. After the last washing the gel is kept in the methanol–water–acetic acid mixture for 1–2 h. Proteins which do not give stable complexes with amido black may be stained with bromophenol blue, light green or other dyes well-known in paper electrophoresis. Staining may also be carried out with a saturated solution of Buffalo black in a mixture of methanol–water–acetic acid (50:50:1) for 2 min. The solvent is used as washing fluid. After removal of the excess of stain the strips are left overnight in the solvent[100].

Another technique includes fixation of the gel in 5 % acetic acid before staining. In this case the colouring mixture consists of 1 g amido black 10B, 500 ml 1 *M* acetic acid and 500 ml 0.01 *M* sodium acetate. The strips are washed in 5 % acetic acid[6].

According to NEELIN AND CONNELL[101] nigrosine stains more dilute components more heavily than does amido black. However, it obscures fine zones in a densely stained region.

Lipoprotein may be stained in a solution containing 1.2 g Sudan black, 40 ml water, 600 ml ethanol and 2 ml NaOH for 16 h. Complete decoloration takes 6–8 days in 60 % alcohol[102]. A saturated solution of oil red O in a mixture of methanol–

acetic acid–water (60:10:30) may also be used in order to make lipoprotein visible[103].

Copper-containing proteins, such as haemocyanin, are detected by placing the horizontally sectioned gel for 3–4 h in a solution containing 50 ml 10% sodium acetate and 3 ml 0.1 % solution of dithio-oximide in alcohol. A positive test results in the development of a greenish black colour[104].

Haemoglobins are detected with the benzidine reagent according to FRANKLIN AND QUASTEL[105] or as described for agar electrophoresis[106] but without zinc acetate solution. Haem proteins may also be stained by *o*-tolidine[107].

The $a_2$ region may be made visible by adding haemoglobin to serum protein. The result is a faintly coloured band which makes the detection of the $a_2$ region possible without staining[108].

OWEN *et al.*[109] use another colour reaction suitable for the detection of small amounts of haptoglobins in human serum. The complex of these proteins with haemoglobin shows peroxidase-like activity. *o*-Dianisidine appeared to be the best reagent, giving a stable brownish colour, whereas it was not absorded on peroxidase-free gel.

### 8. *Quantitative evaluation of the strip*

FINE AND WASZCZENKO[110] prepared gels suitable for direct photometric estimation. Since several protein dyes in alcoholic solution cause permanent opacity of the gels these authors used the aqueous staining method of URIEL AND GRABAR[111].

The strips are fixed overnight in 5 % acetic acid and then stained for 10 min. Decoloration is achieved by successive washings in 5 % acetic acid.

The stained gel is placed on a glass plate covered with 1.5 % melted agar and dried in an oven. The glass plate is withdrawn after the agar has solidified. A sheet of Whatman No. 1 filter paper is applied to the surface. The gel enclosed in agar is left in an oven at 37° for 40 h. In this way a transparent film is obtained.

A simpler method is described by VESSELINOVITCH[100]. Following staining, the strips are immersed in a 10 % solution of acetic acid and boiled for 30–60 sec. After the solution has cooled the strips may be removed and estimated photometrically.

GOLDBERG[99] applied densitometric scanning of photographs of the strips in order to obtain quantitative evaluation of the starch pattern.

The coloured bands producedby some dyes fade on storage of the gels. JACOBS[150] describes a photographic routine method which allows permanent recording of the strips.

### 9. *Elution*

After the electrophoretic run and staining of one of the strips cut horizontally, the uncoloured half may be cut vertically into segments of 0.5 or 1 cm width. Comparison with the stained strip makes location of the separated protein fractions possible.

The protein may be removed from the gel by freezing and thawing of the segments followed by extraction with distilled water or buffer. This procedure does not allow quantitative elution of the substance, as does the starch block technique.

Another more complicated method is digestion of the starch with amylase before protein elution.

## APPLICATIONS

### (*a*) *Serum proteins*

Starch gel electrophoresis allows the identification of more haemoglobin fractions than do other electrophoretic techniques. ROSA *et al.*[112] observed a difference in haemoglobin pattern in six inbred strains of mice.

DE GROUCHY[113,114], who originally used the borate buffer system of SMITHIES, studied the behaviour of normal and abnormal human haemoglobin in a discontinuous buffer system (Tris–citrate–borate). Better solution was obtained and minor components, not detected by other types of electrophoresis, were observed.

GOLDBERG[99] also used a discontinuous buffer system in analyzing human serum haemoglobin.

BANGHAM AND BLUMBERG[115] studied the distribution of electrophoretically different haemoglobins between cattle breeds from Europe and Africa. There was a certain correlation between the absence of bovine B haemoglobin and a relatively high tolerance to trypanosomiasis.

Haptoglobins ($= \alpha_2$-globulins) combine specifically with oxyhaemoglobin. The complex appears to be relatively stable, while uncombined oxyhaemoglobin is rapidly broken down with the formation of methaemalbumin. From these observations ALLISON AND REES[116] concluded that haptoglobins determine the renal threshold for haemoglobin. These authors ascribe haemoglobinuria to low levels or absence of haptoglobins in plasma.

Genetically controlled variations in haptoglobins of human sera were demonstrated by SMITHIES and co-workers[6, 117, 118].

Using borate buffer ASHTON[119] failed to detect similar differences in other mammalian sera. However, using phosphate buffer, it could be demonstrated that differences in cattle serum protein patterns were under genetic control[120]. In a preliminary communication[121] ASHTON described five serum $\beta$-globulin phenotypes of cattle. A sixth phenotype was recognized later[122] so that it was possible to propose a genetic mechanism for the system. The same author observed 3 $\beta$-globulin types, 5 $\alpha$-globulin types, and at least 3 albumins in horse sera[123].

FINE *et al.*[124, 125] found similar patterns to those of SMITHIES but the $\gamma$-globulin was distinctly divided into two components. The distribution of zones in the electrophoretic pattern was as follows: 2 $\gamma$-globulins on the cathodic side of the origin. Staining with Sudan black revealed the presence of $\alpha$- and $\beta$-lipoprotein on the anodic side. Furthermore, an S-$\alpha_2$ band, 9 zones between S-$\alpha_2$ and the $\beta$-c-globulin, 2 F-$\alpha_2$-globulins and 5 zones between the fast $\alpha_2$ and the albumin were detected by the usual staining method.

HARRIS *et al.*[126] demonstrated in a series of 153 sera from Africans new $\beta$-globulins. According to SMITHIES[6, 117, 118] differences in these globulin patterns are controlled genetically. The author referred to the new globulins as $\beta$-CD$_1$ and $\beta$-CD$_2$. $\beta$-Globulin polymorphism in humans distinguishes the white and the black races[127]. So sera from 425 normal adult blood donors were examined by the two-dimensional method in order to demonstrate the absence of $\beta$-globulin D in whites. One-dimensional starch gel electrophoresis was not always reliable for detecting this protein[128].

ASHTON[129] obtained 8 $\beta$-globulins in sheep sera. Further separation of the zones detected resulted in 14 $\beta$-globulin phenotypes. According to that author, sheep sera show the most complex $\beta$-globulin polymorphism ever found in mammals[103].

SMITHIES AND POULIK[97] obtained originally more than fifteen resolved components from serum proteins by two-dimensional electrophoresis. In a later communication[131] the occurrence of more than twenty components was reported.

WOODS *et al.*[132] demonstrated different haemocyanines in sera of certain crustaceous species.

MOULLEC AND FINE[133] who examined the sera of 406 blood donors claim that the frequencies of the genes $Hp^1$ and $Hp^2$ do not differ very much in 40–60 % of the cases examined. This conclusion holds for most of the populations living in Europe and the white population of the U.S.A.

LATNER *et al.*[134] demonstrated that the major binding of vitamin $B_{12}$ by serum proteins occurs by the $\alpha$- and $\beta$-globulins.

FINE AND CREYSSEL[135] studied the electrophoretic behaviour of abnormal proteins in myeloma and macroglobulinaemia. A typical $\beta$-myeloma showed three abnormal components as compared with normal sera. However, macroglobulins gave no characteristic pattern.

FINE AND BURSTEIN[102] fractionated human serum lipoproteins. The most important component migrated between the origin and $\alpha$-globulin. According to the authors the small mobility is caused by a high molecular weight of the lipoprotein fraction.

DE GROUCHY[136] described a method for identification of caeruloplasmin in human serum. The zone was detected by incubation with a solution of *p*-phenylenediamine. The caeruloplasmin coincided with F $\alpha_2$-globulin and was absent in sera of Wilson's disease.

$^{131}$I-Thyroxine added to human serum was run on starch gel. The thyroxine-binding protein migrated ahead of the albumin in a sharp band in the first pre-albumin position[137].

### (*b*) *Other proteins, enzymes, and hormones*

HOERMAN[138] separated parotid saliva proteins. Reproducible migration patterns were obtained in which 9–12 components were resolved. An advantage was that only small volumes of native parotid secretion were required.

PERT AND KUTT[103] observed in normal cerebrospinal fluid 10–12 protein components corresponding to the serum protein in the same individual but with different relative concentrations. In neurologic diseases considerable alterations of the protein pattern were noted.

Starch gel electrophoresis of *Azobacter* proteins[139] revealed the presence of 12 components, differing in amino acid composition.

NEELIN AND CONNELL[101] applied starch gel electrophoresis in combination with cation exchange chromatography to chicken erythrocyte histone. Both mobility and resolution were dependent upon ionic strength, the nature of anions, pH, and on the protein concentration.

POULIK demonstrated the complex nature of diphtheria toxins[140]. In paper electrophoresis an apparent homogeneity was observed. Furthermore the lethal factor from these toxins could be isolated [141].

DIXON AND SMITHIES[142] separated enzymes from cabbage juice. Initially the enzymes showed peptidase activity towards leucyl and glycyl peptides and glycyl transfer activity. It was more difficult to fractionate these activities by other methods.

MARKERT AND HUNTER[143] separated the esterases in B2 mouse tissues. These esterases were compared, using $\alpha$-naphthyl butyrate as a substrate.

TANG *et al.*[144] studied the properties of crystalline gastricsin. When electrophoresis of the enzyme was carried out on paper and starch under similar conditions, it only migrated on starch gel. Furthermore no activity could be recovered from paper strips, probably on account of strong adsorption or denaturation. From the gel the proteolytic activity could be extracted.

PIERCE and coworkers[145] purified thyrotropin. The hormone was separated into diverse fractions after chromatography on DEAE cellulose. One of these fractions was submitted to starch gel electrophoresis and appeared to split up into six fractions These findings were in contrast to results obtained with paper and moving-boundary electrophoresis.

### (*c*) *Non-protein substances*

Except in one instance, starch gel electrophoresis has hitherto not been utilized in the separation of organic substances other than proteins.

FRANGLEN AND GOSSELIN[146] obtained a very remarkable result. These workers succeeded in separating metastable polymers of the dye bromocresol green. From this investigation it appeared clearly that the presence of two distinct bands after the electrophoretic run in starch gel is not always an indication of heterogeneity.

## CONCLUSION

Numerous investigations have demonstrated without doubt that—from an analytical standpoint—the starch gel technique has certain advantages over the starch column method, and even over the starch block method. For preparative purposes, however, the gel technique has up to now not been found convenient. There must be certainty that the separated fractions are homogeneously distributed through a cross section of the medium. In starch gel strips the separation is never completely homogeneous throughout the gel when the strip is sliced after the electrophoretic run. For analytical purposes this is of no importance, as only the stained surface of the strip cut horizontally is considered. In preparative work, however, the variations in different layers of the gel cause overlapping of the separated zones and the stained surface does not correspond with the pattern inside the strip.

In several cases starch block electrophoresis appears to combine the remarkable resolving power of the gel technique with the possibility of isolating the separated fraction after the electrophoretic run. So BEARN AND FRANKLIN[147] made a comparison of starch gel and starch block patterns of the haptoglobin–haemoglobin complexes from the three main genetic groups. Starch block electrophoresis also enabled a distinct differentiation of the three groups to be made. This was important for the isolation of sufficient material for ultracentrifugal studies.

Woodin[148] submitted partially purified leucocidine from *Staphylococcus aureus* to electrophoresis in starch gel. Complete recovery of the protein was not obtained. On the other hand, nearly quantitative recovery was observed with the starch block technique.

In a recent paper Miller and Bernfeld[149] claim that a combination of starch granules and starch gel has the advantages of both media. This important finding awaits further verification.

Summarizing, it may be stated that starch gel electrophoresis is a powerful analytical tool, chiefly for the study of proteins. The utmost attention must always be paid even to the smallest detail of an obtained pattern. As a result, the success of the method depends to a great extent on the experience of the worker.

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# COILED COLUMNS AND RESOLUTION IN GAS CHROMATOGRAPHY*

J. CALVIN GIDDINGS
*Department of Chemistry, University of Utah, Salt Lake City, Utah (U.S.A.)*

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There are a large number of ways in which nonequilibrium effects can arise in chromatography, and each serves to increase the height equivalent to a theoretical plate ($H$). The best known sources of nonequilibrium are in the adsorption or partitioning processes themselves[1]. However, nonequilibrium will be found in general whenever molecules can exist in different states traveling with different velocities, and which convert into one another at a finite rate. Molecules in the mobile and in the stationary phases represent these states in the ordinary case. In the case of coiled columns, used particularly in gas chromatography, molecules near the inside are traveling at a greater velocity than those near the outside, leading to a nonequilibrium laterally across the column. Nonequilibrium of this type also arises as a result of wall effects and column inhomogeneity[2]. This effect can easily be calculated for coiled columns under the assumption that the departure from equilibrium is not large. The method used for this calculation is an extension of that used to describe nonequilibrium between the stationary and mobile phases[3]. The method can be used for nonequilibrium effects in general.

Let us assume that a column has a circular cross section of inside radius $r_0$ and that the packing is essentially homogeneous so that no other nonequilibrium effects arise. The column is bent with a radius of curvature at the center of the tube, $R_0$. For the purpose of deriving the equations, $R_0$ does not have to remain constant along the length of the column as it is for coiled columns. We will also assume that the radius of curvature $R_0$ is much larger than the tube radius $r_0$. This simplifies the equations which are obtained.

The mean local carrier velocity $v$ in the tube is related to the velocity $v_0$ at the center by the equation

$$\frac{v}{v_0} = \frac{R_0}{R_0 + r \sin\theta} \cong 1 - \frac{r \sin\theta}{R_0} \qquad (1)$$

where $r$ is the distance from the tube center and $\theta$ is the angle measured from a line extending through the tube center and perpendicular both to the tube direction and to a line joining the tube to the center of curvature. Lateral concentration gradients are established because of the nonuniform character of the velocity, and lateral

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diffusion results from this. The accumulation of solute per unit time and volume due to lateral diffusion is given by

$$s = RD\left(\frac{1}{r}\frac{\partial}{\partial r}r\frac{\partial c}{\partial r} + \frac{1}{r^2}\frac{\partial^2 c}{\partial \theta^2}\right) \tag{2}$$

where $c$ is the overall concentration of solute per unit volume of the column, $R$ is the equilibrium fraction of molecules in the mobile phase, and $D$ is the coefficient of lateral diffusion. The latter quantity, while approximately equal to the diffusion coefficient of the solute, is altered to some extent by the presence of a porous media.

The total rate of accumulation of solute at a given point is given by the sum of all contributions including convection (second term) and longitudinal diffusion (last term)

$$\frac{\partial c}{\partial t} = s - vR\frac{\partial c}{\partial z} + D_z R\frac{\partial^2 c}{\partial z^2} \tag{3}$$

The overall coefficient of diffusion $D_z$, in the tube direction, $z$, is the sum of coefficients due to molecular diffusion, eddy diffusion and the diffusion due to rate effects.

It is now assumed that the departure from equilibrium is not large. Thus the difference between the actual concentration $c$ and the concentration assuming complete lateral equilibration, $c^*$, is small. If we write $c = c^* (1 + \varepsilon)$, then $\varepsilon$ is small compared to unity. This permits us to replace each partial derivative of $c$ by a partial derivative of $c^*$ in eqn. (3).

$$s = \frac{\partial c^*}{\partial t} + vR\frac{\partial c^*}{\partial z} - DR\frac{\partial^2 c^*}{\partial z^2} \tag{4}$$

The overall mass balance equation gives an alternate expression for $\partial c^*/\partial t$

$$\frac{\partial c^*}{\partial t} = -v_0 R\frac{\partial c^*}{\partial z} + DR\frac{\partial^2 c^*}{\partial z^2} \tag{5}$$

Combining this with eqn. (4) we obtain

$$s = (v - v_0)\,R\frac{\partial c^*}{\partial z} \tag{6}$$

Eliminating $s/R$ between eqns. (2) and (6), and using $(v - v_0)$ as obtained from eqn. (1)

$$\frac{1}{r}\frac{\partial}{\partial r}r\frac{\partial c}{\partial r} + \frac{1}{r^2}\frac{\partial^2 c}{\partial \theta^2} = -\frac{v_0 r\sin\theta}{R_0 D}\frac{\partial c^*}{\partial z} \tag{7}$$

Since $c = c^* (1 + \varepsilon)$ and $\partial c^*/\partial r$ and $\partial c^*/\partial \theta$ are zero because $c^*$ is an equilibrium concentration, we have

$$\frac{1}{r}\frac{\partial}{\partial r}r\frac{\partial \varepsilon}{\partial r} + \frac{1}{r^2}\frac{\partial^2 \varepsilon}{\partial \theta^2} = -\frac{v_0 r\sin\theta}{R_0 D}\frac{\partial \ln c^*}{\partial z} \tag{8}$$

The boundary condition on this equation requires that the concentration gradient disappear at the wall, $(\partial\varepsilon/\partial r)_{r_0} = 0$, and furthermore it is required that $\varepsilon$ integrated over the cross sectional area of the tube be zero. The solution to eqn. (8) which satisfies these conditions is found to be

$$\varepsilon = \frac{-v_0 r\sin\theta}{8R_0 D}\frac{\partial \ln c^*}{\partial z}(r^2 - 3r_0^2) \tag{9}$$

The effect of the nonequilibrium shown quantitatively in eqn. (9) can be evaluated by obtaining the material flux, $q$, per unit area averaged over the column or tube cross section.

$$q = \frac{\iint cRvr \, d\theta \, dr}{\pi r_0^2} = \frac{c^\star R \iint vr \, d\theta \, dr}{\pi r_0^2} + \frac{c^\star R \iint v\varepsilon r \, d\theta \, dr}{\pi r_0^2} \tag{10}$$

The range of integration for $\theta$ is 0 to $2\pi$, and for $r$ is 0 to $r_0$. Using eqn. (1) for $v$ and eqn. (9) for $\varepsilon$, and integrating, we obtain

$$q = c^\star R v_0 - \frac{7 v_0^2 r_0^4 R}{96 R_0^2 D} \frac{\partial c^\star}{\partial z} \tag{11}$$

The first term on the right is the flux that would be found if there were no nonequilibrium. The second term on the right is proportional to the equilibrium (average) concentration gradient, and therefore behaves identically to diffusion. The effective diffusion coefficient is

$$D_c = \frac{7 v_0^2 r_0^4 R}{96 R_0^2 D} \tag{12}$$

This parameter describes the smearing of a solute zone due to column bending, and is an effect over and above usual smearing effects. Instead of writing this as an effective diffusion coefficient we can write it in terms of plate height[1] where $H = 2D_c/Rv_0$, or

$$H = \frac{7 v_0 r_0^4}{48 R_0^2 D} \tag{13}$$

These expressions, in common with all nonequilibrium effects, show $H$ to be proportional to $v_0$ and $D_c$ to be proportional to $v_0^2$. The best known example of this dependence is found in the last term of the van Deemter equation.

A result analogous to the above can be derived for tubes with a flat cross section. If $w$ is the distance from the near to the far wall, and $R_0$ is again the distance from the center of curvature to the center of the tube, the nonequilibrium parameter $\varepsilon$ becomes

$$\varepsilon = \frac{-v_0 x}{24 R_0 D} \frac{\partial \ln c^\star}{\partial z} (4x^2 - 3w^2) \tag{14}$$

Using this, as before, to get the flux, we then obtain the effective diffusion coefficient and $H$

$$D_c = \frac{v_0^2 w^4 R}{120 R_0^2 D}, \qquad H = \frac{v_0 w^4}{60 R_0^2 D} \tag{15}$$

These expressions differ only by a constant factor from those for circular cross sections.

The influence of column coiling is not great for the usual dimensions employed in gas chromatography. However, for columns used for preparative work it can be very significant since $H$ increases with the fourth power of the tube radius. Let us calculate the order of $H$ for a column in which $r_0 = 0.3$ cm, $R_0 = 5$ cm, $v_0 = 10$ cm sec$^{-1}$ and $D = 0.1$ cm$^2$ sec$^{-1}$. For this case, which is not atypical, eqn. (13) yields $H = 0.006$ cm or 0.06 mm. A well packed column will yield an overall $H$ something less than 1 mm; hence this effect is of the order of 10 % for the parameters stated.

Now suppose we have a preparative column in which $r_0 = 4$ cm, $R_0 = 40$ cm, and $D$ and $v_0$ are as before. In this case we obtain $H = 2.3$ cm which certainly cannot be tolerated as far as resolution goes. In cases of this kind, where $H$ is calculated to be excessively large, the assumption that the system is near equilibrium is not correct. This does not help the situation, however, since the degree of smearing is of the order calculated except that a great deal of asymmetry will also be introduced into the zone. It would be advisable to always keep such columns straight, for even a single bend through 90° or less would destroy the resolution obtained through careful packing.

## SUMMARY

Equations have been derived for the effect of coiling and bending chromatographic columns. Examples from gas chromatography are used to calculate the plate height due to bending. The plate height is small in most cases, but for the larger preparative columns it is sufficient to annul an otherwise good separation.

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# A SIMPLE ACCESSORY TO A COMMERCIAL GAS CHROMATOGRAPH FOR MICRO-CATALYTIC STUDIES

L. S. ETTRE AND N. BRENNER
*The Perkin-Elmer Corporation, Norwalk, Conn. (U.S.A.)*

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Recently, a new technique has been reported which made use of a microreactor built directly into a gas chromatograph before the chromatographic column for various catalytic studies. This technique offers several advantages:

(a) Because the microreactor is placed directly before the chromatographic column, no special sampling procedure is necessary; the instrument directly analyses the effluent passing the reactor.

(b) It is possible to rapidly survey the activities of a series of catalysts.

(c) Using a flow-type reactor, changes in the activity of the catalyst as reflected in the composition of the reaction products can be investigated as a function of the time using only small quantities of the reactant (reactants).

(d) This technique makes possible the study of many other types of reactions: *e.g.* if an empty heated tube is substituted for the microreactor, thermal or radiation reactions can be followed. In fact any gas–solid reaction which yields volatile products may lend itself to investigation.

Early work in this field has been reported by the Mellon Institute, Pittsburgh, Pa., in cooperation with P. H. EMMETT of Johns Hopkins University, Baltimore, Md.[1–3]. Recent publications [4–8] describing this application have also appeared. In all of these cases, however, specially designed chromatographic equipment was employed.

The purpose of this study was the development of a simple device which makes possible the adaptation of the commercial gas chromatographs to these investigations.

## TECHNIQUE

The device built for this investigation permits two modes of operation: the first of these is a periodic and the second a flow-type reaction, referred to as "slug technique" and "tail-gas technique" (Fig. 1).

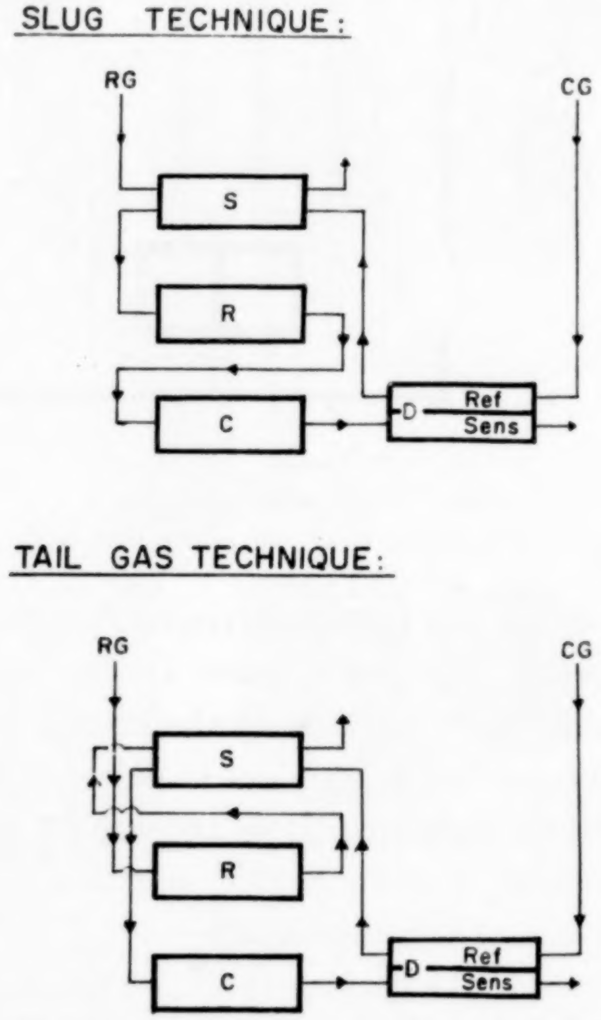
### *Slug technique*

Using this technique, a small sample of the reactant is carried through the reactor by a stream of a carrier gas and the reaction products are conducted directly into the gas chromatograph and analyzed there.

Using this method, only a slug of the reactant passes the reactor. In this case, it is doubtful that an equilibrium could be reached with respect to either the condition of the catalyst or the composition of the reaction products. In many cases this method is sufficient. In other cases, however, the use of a flow-type reactor is necessary.

*Tail-gas technique*

In this case the reactant gas flows continuously through the reactor and the reaction products pass out of the instrument. A special arrangement takes periodic samples from this "tail gas" and conducts them into the gas chromatograph for analysis.


SLUG TECHNIQUE:

TAIL GAS TECHNIQUE:

Fig. 1. Block diagrams of the different microcatalytic techniques. RG: reactant; CG: carrier gas; S: sampling and dosing device; R: microreactor; C: chromatographic column; D: detector cells.

## INSTRUMENT

The equipment built for microcatalytic investigations consists of two parts:

(a) A microreactor device and

(b) a commercial laboratory gas chromatograph, the Perkin-Elmer Model 154.

The Model 154 gas chromatograph is regularly equipped with a special six-port valve to make possible the introduction of a definite volume of gas sample[9-11]. The device built for the microcatalytic investigation is a modification of this "gas-sampling valve".

The schematic of the whole system is given in Fig. 2. As shown, the new device consists of two standard gas sampling valves connected together and installed in place of the regular sampling valve on the side-wall of the instrument. The upper valve is the "sampling valve" and is equipped with the standard sample volume tube

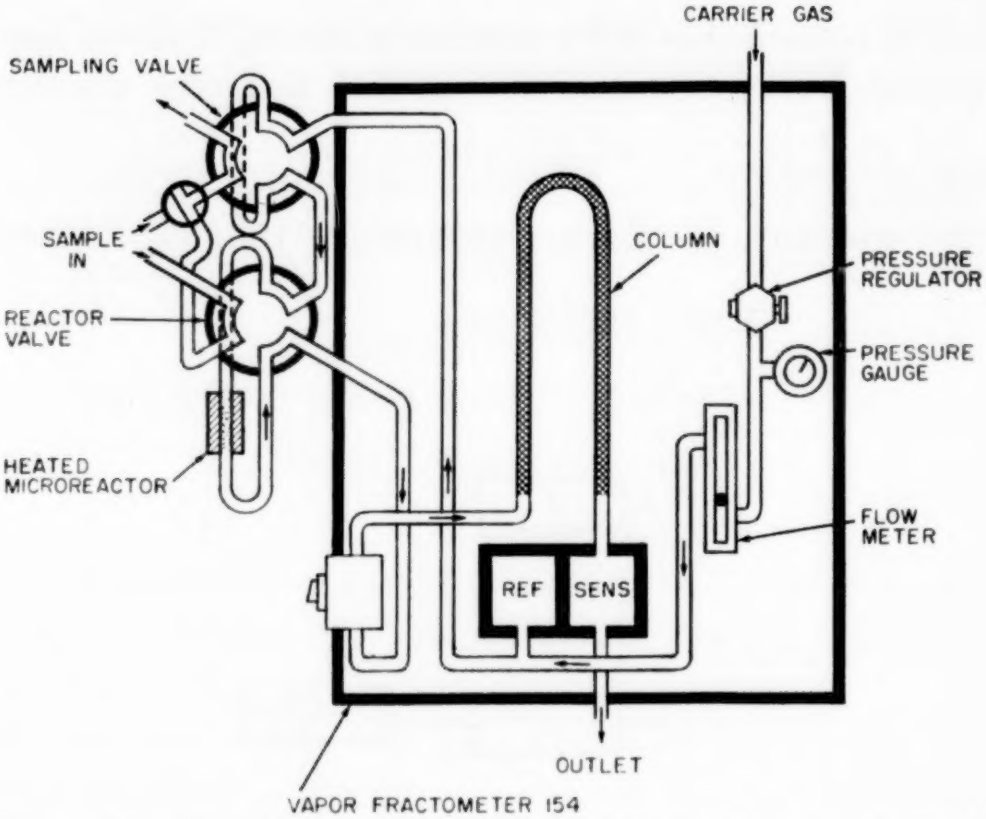


Fig. 2. Flow schematic of the microreactor combined with a gas chromatograph.

and a small three-way valve at the inlet to permit direct analysis of a sample without passing through the reactor. The lower valve is the "reactor valve": the microreactor is connected to this valve, in place of the sample volume tubing.

The microreactor consists of a stainless steel tube (O.D. $^{3}/_{4}$ in., I.D. $^{9}/_{16}$ in., length 4½ in.), connected through two $^{1}/_{8}$ in. tubes with the valve. The reactor tube is contained within a concentric oven heated electrically. A variable heater and a thermometer provide for constant temperature.

The catalyst is placed into the reactor tube and retained with glass wool in order to prevent catalyst from entering the $^{1}/_{8}$ in. connecting tubes. In this particular reactor tube, 1 cm height of the catalyst corresponds to 1.60 c.c.

## PROCEDURE

The equipment conveniently permits operations in three different modes; see Fig. 4(a–c):

### (*a*) *Slug technique*

The sample enters into the second valve, flows through the three-way valve into the sample volume tube of the first valve and leaves it. Turning the first valve to the other position, the carrier gas (helium) flow purges the sample from the sample volume tube through the reactor and then into the fractometer itself.

### (*b*) *Tail-gas technique*

In this mode, the reactant gas flows continuously through the reactor, and, after this, through the sampling tube. The content of this tube is purged from time to time by turning the valve, at which time the tube is purged with the carrier gas flow into the chromatograph and analyzed.

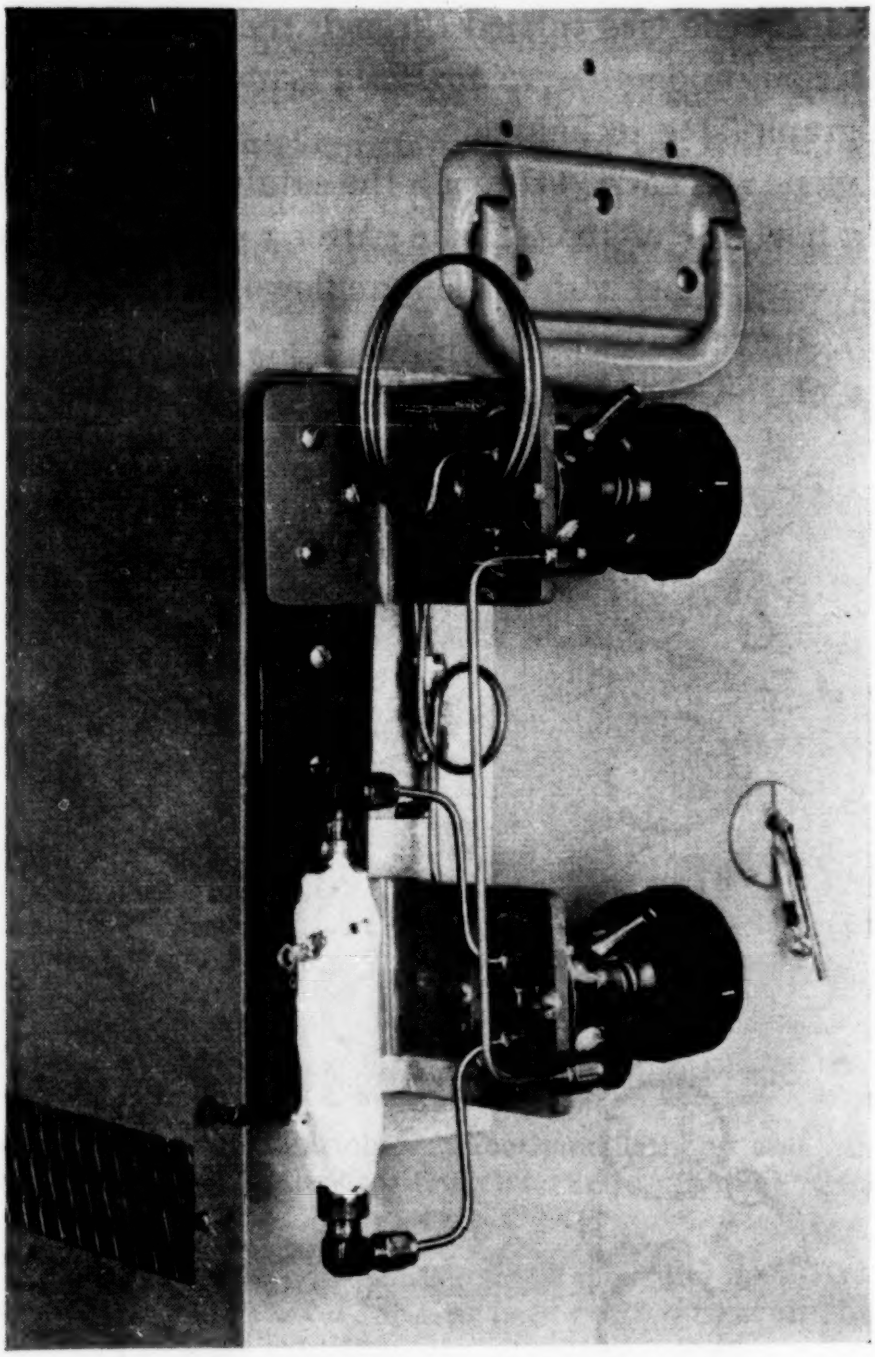


Fig. 3. Photograph of the device.

### (*c*) *Direct analysis of the sample*

In this case, the small three-way valve is turned to disconnect the two valves. The sample inlet is now at the inlet of the three-way valve and upon turning the sampling valve to the "analysis" position, the carrier gas purges the content of the sample volume tube directly into the gas chromatograph. This is similar to the usual function of the unmodified gas sampling system.

## RESULTS

The following investigations show the applicability of the device for catalytic studies.

3.5 c.c. of a typical silica-alumina catalyst* was placed into the microreactor and heated to about 425°. Isobutylene was used as reactant gas and three different investigations were made:

(1) 1 c.c. of isobutylene was analyzed directly with the gas chromatograph (using sampling mode (c) described above).

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* Courtesy of the Esso Research & Engineering Company, Linden, N.J. (U.S.A.).

(2) 1 c.c. of isobutylene was carried through the catalyst bed by the stream of helium and the reaction products were analyzed (sampling mode (a), above) directly with the chromatograph (slug technique).

(3) The isobutylene was carried through the catalyst bed and through the sample volume tube with a flow rate of 100 c.c./min. After 1 min, the content of the sample volume tube (1 c.c.) was purged with the helium flow into the fractometer for analysis (sampling mode (b), above; tail-gas technique).

The conditons of the chromatographic analyses were the following: *column*:

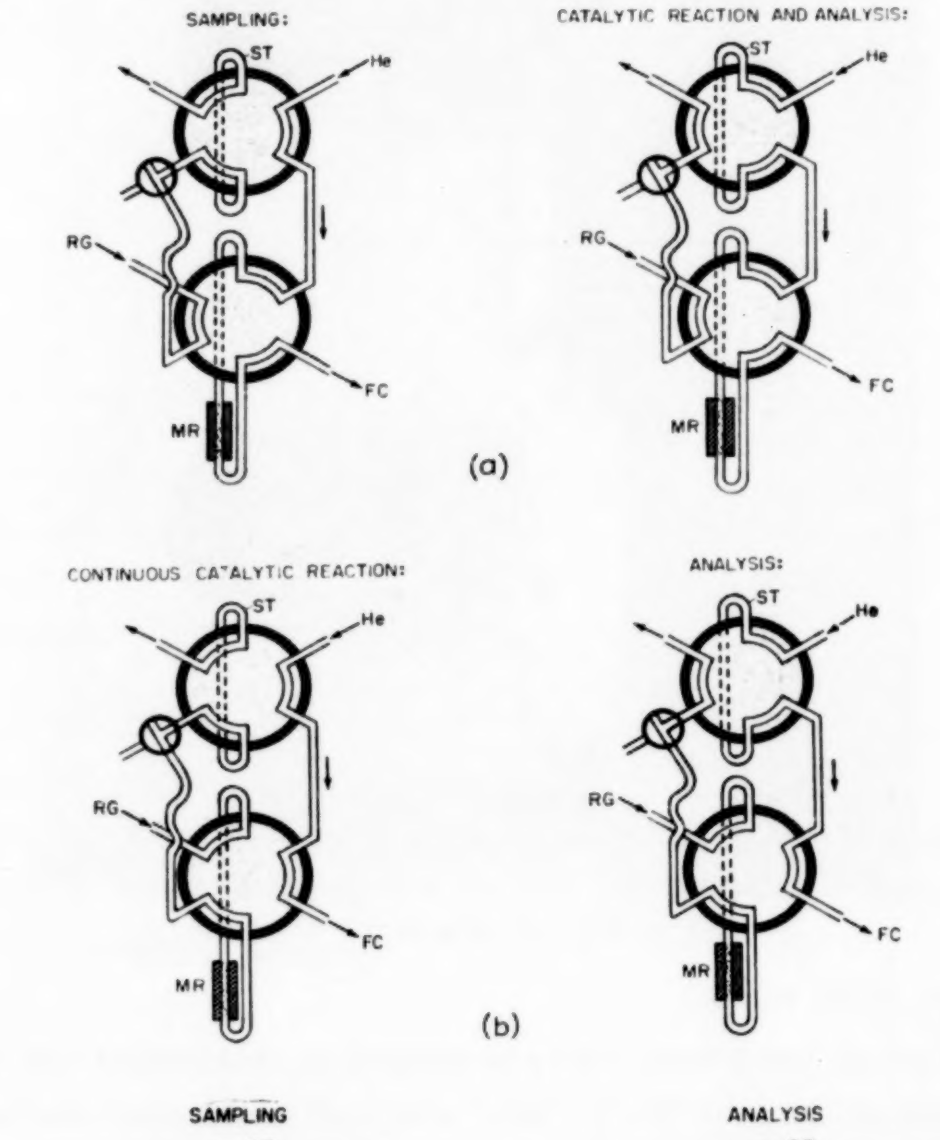
SAMPLING:
CATALYTIC REACTION AND ANALYSIS:
ST
He
RG
FC
MR
ST
He
RG
FC
MR
(a)
CONTINUOUS CATALYTIC REACTION:
ANALYSIS:
ST
He
RG
FC
MR
ST
He
RG
FC
MR
(b)
SAMPLING
ANALYSIS
ST
He
Sample
FC
MR
ST
He
FC
MR
(c)

Fig. 4(a–c). Functional schematic of the sampling and reactor device. RG: reactant gas; He: carrier gas (helium); FC: chromatographic column; MR: microreactor; ST: sampling volume tubing. (a) Periodic examination (slug technique); (b) continuous examination (tail-gas technique); (c) analysis of a gas sample without any catalytic reaction.

4 m dimethyl sulfolane on Chromosorb; *temperature*: 34°; *carrier gas*: helium, 50 c.c./min, 20 psig inlet pressure; *recorder*: 5 mV Leeds & Northrup Speedomax. The sensitivity of the recorder was constant in all of the three analyses.

The chromatograms in Fig. 5 show the results of the investigations. Chromatogram A shows that the isobutylene used was esentially free of any impurities. Using the slug technique, most of the isobutylene was transformed to the other $C_4$-hydrocarbons or cracked to lower homologs. On the other hand, working with the tail-gas technique, the efficiency of conversion was much lower and most of the isobutylene left the reactor unchanged. The reason for this is of course that in this case a relatively high flow of the concentrated reactant gas passes the catalyst as compared with the slug technique.

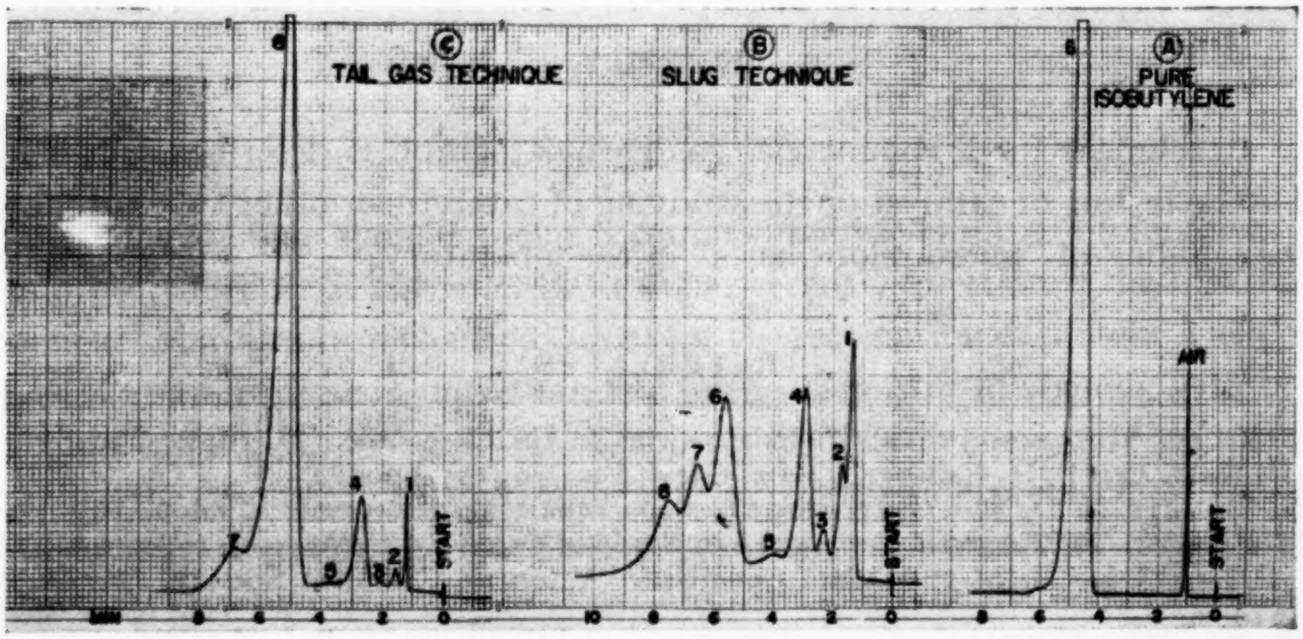

Fig. 5. Chromatograms of the investigations. 1: methane, ethane and ethylene; 2: propane; 3: propylene; 4: isobutane; 5: *n*-butane; 6: isobutylene and butene-1; 7: *trans*-butene-2; 8: *cis*-butene-2.

These experiments show that the new device shows considerable promise in providing means for investigation of catalytic processes on a convenient, small and inexpensive scale.

## FURTHER MODIFICATIONS

### *Other types of reactions*

The device also makes it possible to study other types of reactions. Using a heated, empty reactor tube, thermal reactions could be investigated. On the other hand, with a similar reactor equipped with radiation sources, radiation reactions could be carried out.

### *Liquid samples*

The described device is built for investigations where the reactant is gas or easily vaporized liquid. If *liquid* samples were to be used, the tube between the liquid sample injection block and the chromatographic column could be disconnected and a similar microreactor installed into the system at this point.

### *Automatic operation*

The system described above utilized manually activated valves and a laboratory gas chromatograph. There is however no obvious reason why all operations cannot be performed automatically on a preset timed basis. The substitution of a process-type gas chromatograph for the laboratory version, with suitable valves activated by solenoids and timed by the usual programmer unit would in effect produce an automatically cycling system. The intriguing possibility of constructing a "micro pilot plant" along these lines is thus presented.

## SUMMARY

A new attachment for a commercial gas chromatograph is described which makes possible the testing of catalyst or the studying of catalytic or other types of reactions and the building of "micro pilot plants" in the laboratory.

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# CERTAIN ASPECTS OF BROMOPHENOL BLUE STAINING DEDUCED FROM SPOT TESTS ON FILTER-PAPER

G. G. SELMAN

*Institute of Animal Genetics, Edinburgh (Great Britain)*

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## INTRODUCTION

That bromophenol blue stains proteins is common knowledge; that bromophenol blue can stain proteins only is not true. BAKER[1] has recently questioned the reliability of bromophenol blue as a histochemical agent for the recognition of protein. The present paper aims at an increase in understanding of the chemistry of staining with bromophenol blue. It describes spot tests for organic compounds of moderately low molecular weight, but the results are believed to be relevant to the interpretation of the results obtained by various established methods of staining proteins on filter-paper or starch gel, and they also suggest ways in which bromophenol blue methods could usefully be developed for the histological location of concentrations of specific organic groups.

## EXPERIMENTAL

A wide variety of methods exist for staining proteins on filter-paper with bromophenol blue. In its usual form the staining reagent is made up in ethyl alcohol saturated with mercuric chloride, and contains 1 % bromophenol blue as employed by CREMER AND TISELIUS[2], FLYNN AND DE MAYO[3], and KUNKEL AND TISELIUS[4], or 0.1 % bromophenol blue as used by DURRUM[5], GESHWIND AND LI[6] and HARDWICKE[7]. After this reagent the filter-papers are washed to a white background with tap-water after DURRUM[5] or with 1 % mercuric chloride in methyl alcohol after FLYNN AND DE MAYO[3] and CREMER AND TISELIUS[2], or with 0.5 % (or 2 %) acetic acid as in KUNKEL AND TISELIUS[4] and HARDWICKE[7]. In addition reagents have been used containing bromophenol blue, acetic acid and mercuric chloride in aqueous solution by KUNKEL AND TISELIUS[4] and DURRUM, PAUL AND SMITH[8], and also with the mercuric chloride replaced by zinc sulphate by JENCKS, JETTON AND DURRUM[9]. These were all followed by rinses in dilute acetic acid.

In the present work a series of drops, each of volume one twentieth of a millilitre approximately, of certain compounds in aqueous solution were placed separately on Whatman 3MM filter-paper strips to give a series of spots. The papers were then oven-dried, stained, rinsed and dried again according to whatever procedure was being investigated. After drying the papers were read, and when necessary the visibility of the bromophenol blue spots was enhanced by passing the paper through the fumes of concentrated ammonia immediately before observing. The papers were finally dried, sprayed with 0.2 % ninhydrin in *n*-butyl alcohol, dried again, steamed if necessary and observed. In this way the degree to which amino acids were leached out of the paper into the rising solutions could be followed. The compounds tested were commercial samples of the common amino acids and also included some purines, pyrimidines, vitamins, nucleosides, nucleotides and nucleic acids.

Tests were first made with the standard staining reagent of 1 % bromophenol blue in ethyl alcohol saturated with mercuric chloride used with a staining time of ten minutes. This was followed by washing in steadily running tap-water of pH about 6.5 until a white background was just obtained. This staining method showed a useful specificity, since of the amino acids only histidine and cysteine gave clear blue spots on a white background. The method detected down to 50 $\gamma$ of either histidine or cysteine, and 200 $\gamma$ of these amino acids was detected easily. (Using ninhydrin 12 $\gamma$ of histidine was detected under similar conditions and 25 $\gamma$ was detected easily.) GESHWIND AND LI[6] have already demonstrated this bromophenol blue test for histidine and its peptides, and for peptides of cysteine, although they did not actually test cysteine itself. They also showed that histamine behaves similarly to histidine. They demonstrated that the test depended upon bromophenol blue coupling to the organic compound through mercury to form a complex which is relatively insoluble in the rinsing water. Other amino acids are not retained on the paper. The present work showed in addition that the purines guanine, adenine, hypoxanthine and xanthine gave a positive test, while the pyrimidines uracil and thymine were negative. The purines tested all gave blue spots except guanine which gave the orange colour of the dye in its acid form. Benzimidazole gave a blue spot but uric and orotic acids, the ribose nucleosides xanthosine and adenosine, the nucleotides guanylic, cytidylic and adenylic acids, vitamins $B_2$ and $B_6$, choline and cholesterol, all gave negative tests. From inspection of the structural formulae of these compounds one may conclude that the staining method gives a positive test for imidazole compounds provided that their imidazole ring is not substituted at the position of the nitrogen atom in the nine position, to use the usual numbering for a purine compound. This nitrogen atom is the third atom of the imidazole ring according to FOX[10] and in the unsubstituted compounds there is a double bond between it and the carbon atom two. For imidazole compounds where such a substitution does occur so that the double bond is absent, as in uric acid, xanthosine, adenosine, guanylic and adenylic acids, the test is negative. Tests with DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) gave a very weak blue colour, such as was obtained by MAZIA, BREWER AND ALFERT[11]. The faint dye binding here may be due to Van der Waals forces. The absence of a strong blue colour with nucleic acids is to be expected because of the $\beta$-configuration of their nucleosides, see for example CLARK, TODD AND ZUSSMAN[12].

In addition to the above, the compound aneurine hydrochloride or thiamine, which contains a thiazole ring, gave a very strong blue spot. Indeed this was the only compound which gave a strong blue colour with all the various combinations of reagent and rinses which were tried. It even showed blue while it was in a rinse of 2% acetic acid. Other compounds with thiazole rings have not been tested.

When the same staining reagent was used followed by rinses in 0.5% acetic acid, after KUNKEL AND TISELIUS[4], less amino acid was leached out so that arginine, lysine, aspartic acid and proline were also detectable with bromophenol blue in addition to histidine and cysteine. When rinses in 2% acetic acid were used nearly all the amino acids were retained on the paper to some extent, but the use of the acetic acid made the blue colour difficult to develop even after ammonia fumes. Histidine and cysteine for instance did not give as strong a blue colouration after acetic rinses as when tap-water was used. When rinses of 1% mercuric chloride in methanol were used, after FLYNN AND DE MAYO[3], more amino acid was retained on the paper, as detected by the final ninhydrin spray, and arginine and lysine again gave blue spots with bromophenol blue, but although histidine and cysteine also gave blue spots they were not as easily detectable as with the tap-water rinse.

The method of KUNKEL AND TISELIUS[4] also stained the more basic amino acids but the staining of histidine and cysteine was less intense in comparison.

The method of JENCKS, JETTON AND DURRUM[9], which uses no mercuric chloride, did not give a blue spot with either histidine or cysteine, although a subsequent spraying with ninhydrin showed that these were retained on the paper after the rinses. Arginine, lysine, tryptophan, phenylalanine and proline did give blue spots with this method.

When, however, the staining solution consisted of a saturated solution of bromophenol blue in acetic alcohol (consisting of 3 parts of 95% alcohol to 1 part of glacial acetic acid) and the rinsing was done in 0.5% acetic acid, blue spots were given by arginine, lysine, thiamine and histidine but not cysteine. Presumably the staining of histidine in this case was through its amino group.

## DISCUSSION AND CONCLUSIONS

Now the above staining methods were all originally developed for heat-coagulated proteins on filter-paper after separation by paper electrophoresis. In the tests described above, whatever the method of rinsing, there was a greater loss of amino acid during the rinsing procedure than would be the case with proteins stained in the same manner. Nevertheless, since the amino acid composition of each protein is different, then it follows from the above observations that proteins will differ in their bromophenol blue staining intensity per gram of nitrogen. This has been shown directly by JENCKS, JETTON AND DURRUM[9] for the electrophoretically separable protein constituents of serum. Of course proteins may also differ in the availability of their staining groups. In addition the present observations indicate that each different staining procedure will be expected to give a different staining intensity for the same protein. Several authors have described their particular methods as quantitative, for example, FLYNN AND DE MAYO[3], KUNKEL AND TISELIUS[4] and JENCKS, JETTON AND DURRUM[9], but unless correction factors are applied, firstly for the individual proteins or classes of proteins and secondly for the staining and rinsing procedures used, no quantitative comparison can be made between different sets of results.

The method of GRASSMANN AND HANNIG[13], used by FLYNN AND DE MAYO[3], employs Naphthalene Black as a stain for proteins on filter-paper. Using a sample of Naphthalene Black 12B 200 from Imperial Chemical Industries Ltd., in exactly the same manner as the latter authors, it was found that all the amino acids, purines, pyrimidines etc. gave darker blue spots on a pale blue background. It was not found possible to detect less than 250 $\gamma$ of histidine with this method because the blue background limited sensitivity. However, since the method detects a wider range of compounds than does ninhydrin for example, it has obvious value for detecting compounds whose chemical natures are unknown. Its lack of specificity suggests also that Naphthalene Black may be superior to bromophenol blue for semi-quantitative work when comparisons are required between proteins of widely different amino acid composition. Amidoschwarz 10B is the same dyestuff as Naphthalene Black 12B or 10B.

Bromophenol blue has been used as a histological stain by MAZIA, BREWER AND ALFERT[11]. For histochemical purposes it is useful to remember that this dye can couple to the imidazole ring of histidine and to the sulphydryl group of cysteine, provided that mercuric chloride is present. For simple acid staining of the more basic amino acids and for the case of thiamine it does not matter whether mercuric compounds are present or not. It is easy to see how proteins are stained in these ways, but it also follows that other compounds which are not proteins but which contain sulphydryl groups, imidazole rings which have not been substituted in the manner described above, amino groups, or perhaps thiazole rings, may also stain. MAZIA and his colleagues suggested that coupling to free carboxyl groups via mercury may also occur but no case of this was noticed in this work. Of course, after hot trichloracetic acid extraction (see *e.g.* TAFT[14]) there may be little left to stain except protein, but interesting cytological results are usually obtained by less drastic methods. It is suggested that if the amino groups are first blocked or changed, then bromophenol blue with mercuric chloride could be used to stain for the sulphydryl groups and histidine, or for either separately by the use of further blocking reagents. Guidance in the choice of appropriate blocking reagents for achieving this, and for confirming the results, is obtainable from a study of DANIELLI[15], BOWYER[16] and MUNDAY AND GIERER[17].

## SUMMARY

Spot tests are described using bromophenol blue for low molecular weight organic compounds of biological importance. One method for carrying out the spot test detects sulphydryl groups, imidazole rings provided that they are not substituted at a particular nitrogen atom, and thiamine. A comparison of the different ways of using bromophenol blue leads to conclusions relevant to the use of the dye for staining proteins etc. on filter-paper or starch gel and its use in histochemistry.

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# ON THE INTERDEPENDENCE OF PROTEIN DIFFUSION, ELECTROPHORETIC MOBILITY AND GEL STRUCTURE

CH. WUNDERLY

*Medical University Clinic, Zurich (Switzerland)*

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Since GORDON, KEIL AND ŠEBESTA[1] succeeded in 1949 in fractionating proteins in a 1% agar gel, this support has found a wide application for electrophoretic work. When electrophoresis is followed by gel diffusion of the specific antibody we have immuno-electrophoresis, as developed by GRABAR AND WILLIAMS[2] in 1953; the method has become a valuable tool for clinical and immuno-chemical investigation, since it possesses the increased sensitivity of the immunological techniques. Using double diffusion of antigen and of antibody in an agar gel[3], the presence or absence of immunologically specific antigen antibody systems can be demonstrated. Agar gel electrophoresis found new applications: GIRI[4] introduced a two-dimensional technique and WIEME AND RABAEY[5] developed ultramicroelectrophoresis on a quantitative basis; the latter gives good resolution starting with 0.1 ml of a protein solution of 0.02% (0.02 mg of protein). Other studies[6] have reduced the agar concentration to a point just below its gelling concentration. Since an agar gel of 1% still shows sufficient rigidity, 99% of the gel is fluid and allows for conditions which equal those of "free" electrophoresis; in comparison with starch as a support medium, agar has the disadvantage of a higher adsorption for proteins and a strong electroosmotic flow.

Though numerous symposia[7] have collected reports of investigations on diffusion and immunological reactions in agar gel, only a few papers[6,8] have dealt with the colloid-chemical side of these phenomena. The aim of the present contribution is to demonstrate the correlation of gel structure with diffusion and electrophoretic mobility of proteins. In particular, the influence of carbohydrates on the structure of the agar gel is dealt with.

## METHODS

### (1) *Gel diffusion*

On glass plates of 4 × 16 cm, as used with the Elphor-apparatus for paper electrophoresis, a gel is formed with 12 ml of 1.5% agar (Difco Bacto Agar) in veronal acetate buffer of pH 8.6 and $\tau/2 = 0.1$. On this gel—the basis gel—an intermediate gel of 1.8 mm thickness is laid[9]; it contains 0.666% agar and various carbohydrates capable of influencing gel structure (see Table II). In order to estimate the correlation of molecular weight and particle size with diffusion velocity, three protein solutions containing 2% of serum albumin* (human), 2% $\gamma$-globulin* (human) and $\alpha_2$-macroglobulin*, are prepared. These solutions are taken into the Agla pipette; 6–7 drops containing 200 $\mu$g of a fraction are placed at regular intervals on the surface of the intermediate gel, which has been prepared 1 h previously. After 48 h in a wet chamber at 20° these intermediate gels are gently stripped off and laid on a glass plate of 4 × 16 cm. Together with the basis gels they remain for 3 h in 2% acetic acid; later they are covered with a wet filter paper, dried under infrared light and stained with Naphthalene Black 12B 200[10]. Two gel plates are always run in parallel; these allow for 12–14 stained spots of protein, which are then measured in the Zeiss Extinktionsschreiber II; the smallest and the largest values are attributable to gel heterogeneities and are ruled out; the remaining values must not differ by more than 5% (see Fig. 1).

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* Central Laboratory of the Swiss Red Cross, Berne.

### (2) *Electrophoresis*

On glass plates of 4 × 16 cm a gel is formed with 12 ml 1.25% agar (0.19 ml/cm$^2$; thickness of gel 1.9 mm) in the above-mentioned buffer of pH 8.6. All the runs are made in duplicate and last 6 hours. The current is switched on 30 min before the proteins are placed on the gel. A mixture is prepared containing 2% serum albumin and 2% $\gamma$-globulin; a drop from the Agla pipette, containing 200 $\mu$g of each fraction, is placed on the centre of the agar gel. After an interval of 15 min to allow the liquid to diffuse into the gel, the current is switched on. The voltage is stabilized by the Reco model E-800-2** Powerstat. In order to determine the electroosmotic flow, a drop of a 16% solution of PVP (Plasdone) is placed on the centre of the agar gel. After the run this gel is covered with a strip of moist filter paper Munktell 20/150 and dried under the infrared lamp; the paper takes up the PVP. It is then dyed for 5 min with bromophenol blue and washed with water, when the position of the PVP spot is revealed. The distance travelled by the PVP spot from the starting line is added to the distance of protein anions and subtracted from the cations; thus $\mu$ is arrived at in cm$^2\cdot$sec$^{-1}$ V$^{-1}$. The proteins in the agar film are stained for 10 min in a solution of methanol, saturated with Naphthalene Black 12B 200 and containing 10% acetic acid. Subsequent washing is carried out with methanol + 10% acetic acid, changed three times. This technique allows for *independent measurements of electrophoretic migration velocity as well as electroosmotic flow in the same run.*

### (3) *Specific conductance*

The resistance $v$ of the agar gel between the electrodes is expressed by the equation

$$v = \frac{V}{A} = \sigma \cdot \frac{l}{q}$$

where $l$ is the length between the electrodes, 12.5 cm, and $q$ the surface of a cross-section, 0.072 cm$^2$. The specific conductance $\varkappa$ equals the reciprocal specific resistance $\sigma$.

$$\varkappa = \frac{A}{V} \cdot \frac{l}{q} = \text{Ohm}^{-1} \cdot \text{cm}^{-1}$$

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* Behring Werke, Marburg/Lahn, Germany

** Research Equipment Corp., Oakland, Calif.

with the current intensity $A$ in mA and the voltage in V. Electrolytical conductivity is generally an exponential function of the absolute temperature; therefore all runs were made at a constant temperature of 20°.

(4) *Colloid chemistry of the gel*

(*a*) *Viscosity*. All measurements were done with the Ostwald capillary viscosimeter in a constant temperature bath at 70°. In order to correlate the specific viscosity $\eta_{sp}$ with concentration $C$, $\eta_{sp}$ is divided by $C$. The resulting viscosity number or index $\eta_c$ is then

$$\eta_c = \frac{\eta - \eta_0}{\eta_0 \cdot C} = \frac{\eta_{sp}}{C}$$

(*b*) *Turbidity* is measured in the Zeiss Step Photometer with a special attachment for the measurement of stray light under an angle of 45°. The sols were measured at 70°, the gels at 20° 2 h after setting. The turbidities are given in percentages of the standard turbidity No. 4, which is taken as 100; our data are thus relative turbidities but comparable.

(*c*) *Tarr-Baker test*. This test was developed in 1926 for testing "jelly strength"[11-14]. A cylindrical glass stamp of 20 mm diameter is slowly pressed into the gel; when the ultimate strength is passed, the surface cracks and the stamp breaks into the gel. The critical pressure is given by the length of a $CCl_4$-column in cm. This length multiplied by 1.6 gives the length of a corresponding water column in cm (see Table III). Gels are tested 7 h after they have set.

(*d*) *Säverborn test*. By this test the rigidity of the gel can be measured[15,16]. The gel is formed in the space left between an inner and an outer metallic cylinder. On the former a constant force is acting to turn the inner cylinder in the gel. The counteraction of the gel gives a measure of its torsional strength; it is measured optically and expressed in dynes per cm$^2$ (modulus of rigidity). Gels are measured 7 h after they have set[17-19] at a temperature of 18°. The gel is covered in order to minimize evaporation.

## RESULTS

*Diffusion*

In order to obtain comparative values of stained proteins without diffusion, 7 drops containing 200 μg of serum albumin were placed by means of the Agla pipette on a 1.5% agar gel 1.8 mm thick. Two hours later the gel was dried and stained with Naphthalene Black. The same was done with γ-globulin and with $\alpha_2$-macroglobulin. For each protein 20 spots were measured by the Zeiss Extinktionsschreiber II and the results compared statistically ($n = 20$).

For the following studies of gel diffusion the surfaces quoted in Table I, column 4, are standard values (100%); the surfaces found after diffusion through the intermediate gel (*cf.* METHODS (1)) are given in percentages of the standard (Table II). In Fig. 1 the surfaces of stained spots of protein are depicted as they are delivered by the Zeiss apparatus.

Contrary to the experience on cellulose[22,23] γ-globulin takes a slightly more intense stain (Naphthalene Black 12B 200) than serum albumin.

The colloid-chemical data of these intermediate gels are given in Table III. Early electrophoretic measurements had shown that the results in 0.666%

TABLE I

| *Protein fraction* | *Mol. wt.** | *Length and diameter** *Å* | *Diffusion constant* $D_{20} \times 10^{-7}$ *cm²/sec* | *Surface of stained spots (average value) cm²* | *Standard deviation* $\sigma$ | *Deviation of the average value* $\sigma/\sqrt{n}$ | *Average error* $\sigma^2/\sqrt{n}$ |
|---|---|---|---|---|---|---|---|
| Serum albumin | 69,000 | 150 × 38 | 6.1 [25] | 1.15 | ± 0.060 | 0.0166 | 0.00028 |
| $\gamma$-Globulin | 152,000 | 235 × 44 | 3.6 [26] | 1.60 | ± 0.076 | 0.0197 | 0.00038 |
| $\alpha_2$-Macroglobulin[24] | 846,000 | — | — | 0.75 | ± 0.074 | 0.0208 | 0.00043 |
| Plasdone** (PVP) | 90,000 ± 15,000 | 350 × 18 | — | — | — | — | — |

* From sedimentation and diffusion measurements[20,21].
** Antara Chemicals, New York, N.Y., U.S.A.

TABLE II

| *Intermediate agar gel of 0.666%* | *Diffusion into basis gel after 48 h* | | |
|---|---|---|---|
| | *Albumin* % | *γ-Globulin* % | *$\alpha_2$-Macroglobulin* % |
| Agar gel, 0.666% | 47 | 38 | 30 |
| Additions: | | | |
| 3.33% Sucrose | 65 | 43 | 38 |
| 3% Dextran L* | 42 | 40 | 15 |
| 0.83% Dextran H* | 19 | 30 | trace |
| 0.025% Carrageenan** | 9 | 19 | trace |

* R. K. LAROS, Bethlehem, Pa. U.S.A.
** Institute for Agricultural Chemistry of the Federal Polytechnical School, Zürich (Prof. Dr. H. DEUEL).

TABLE III

| *Gel composition* | *Mol. wt. of additions* | *Viscosity* $\eta_{sp}$ | *Turbidity rel.%* | | *Tarr-Baker test (gel strength) cm $H_2O$ column* | *Säverborn test (rigidity) dynes/cm²* |
|---|---|---|---|---|---|---|
| | | | *Sol* | *Gel* | | |
| Agar gel, 0.666% | — | 0.76 | 21 | 225 | 138 | $6.4 \cdot 10^3$ |
| Additions: | | | | | | |
| 16.66% Ethylene glycol | 62 | 1.58 | 17 | 178 | 160 | $7.1 \cdot 10^3$ |
| 3.33% Sucrose | 349 | 1.19 | 16 | 185 | 170 | $6.3 \cdot 10^3$ |
| 3.00% Dextran L | 15,000– 20,000 | 1.18 | 20 | 250 | 159 | $11.0 \cdot 10^3$ |
| 0.83% Dextran H | 90,000–100,000 | 1.38 | 60 | 232 | 159 | $7.6 \cdot 10^3$ |
| 0.10% Carrageenan | approx. 300,000 | 3.29 | 18 | 192 | 216 | $10.8 \cdot 10^3$ |
| 0.025% Carrageenan | | 1.57 | 14 | 138 | 181 | $8.6 \cdot 10^3$ |

agar-gel, supplemented with the additions indicated in Table II, do not reveal significant changes in migration velocity. Therefore the following experiments were carried out in 1.25 % agar gel, with a field strength of 1.60 V/cm and a current intensity of 2.25 mA/cm. Duration of electrophoresis was 6 h. The temperature in the gel did not rise above 20°. Evaporation was minimal since the gels were covered with a sheet of wet porous rubber[6]; their loss in weight remained below 4 %.

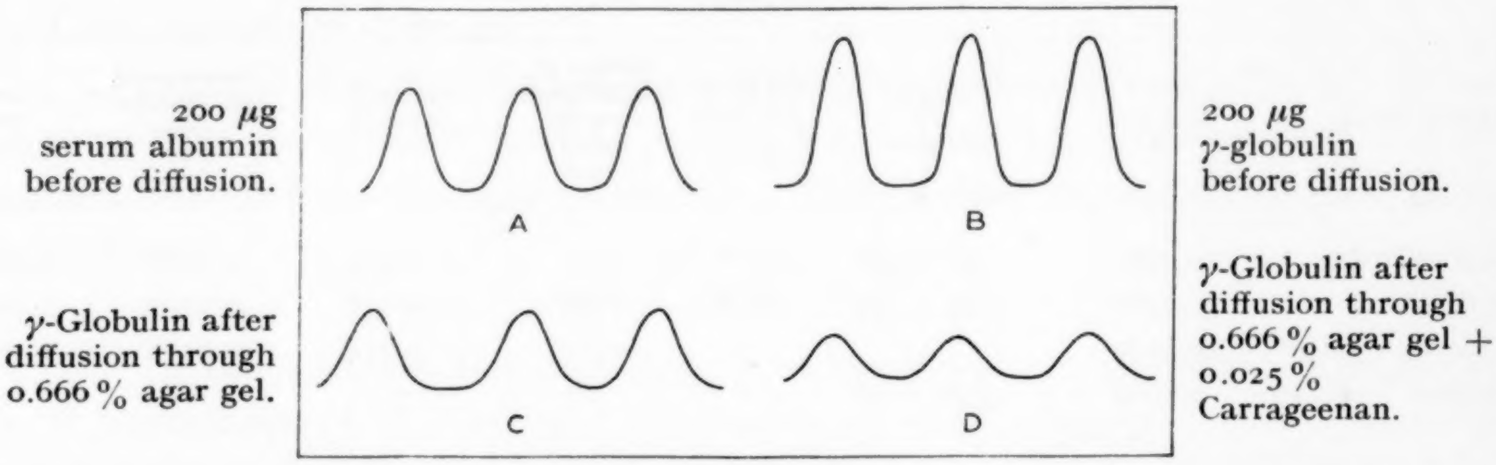

Fig. 1. Curves of stained protein spots before and after diffusion, as given by the Zeiss Extinktionsschreiber II.

Gels containing ethylene glycol and Dextran H adhered so strongly to the paper, when drying, that the staining was much impaired.

Since the addition of 0.10 % Carrageenan to agar gel causes extremely high values for viscosity, gel strength and rigidity (see Table III), the rate of electrophoretic migration was measured in gels of 1.25 % agar in a buffer of pH 8.6, τ/2 = 0.1 and simultaneously in gels of 1.25 % agar plus 0.1 % Carrageenan. These gels were formed on glass plates 10 × 15 cm; they contained 0.24 ml/cm$^2$ and were 2.4 mm thick. The size of the gel allowed 6 electrophoretic runs under strictly equal conditions of temperature and evaporation. Each run was done three times; reproducibility of results lies within ± 5 %. The field strength was kept constant at 2.26 V/cm and the current intensity at 4.00 mA/cm. The rate of migration and of electroosmosis was determined after 2, 4 and 6 hours.

## TABLE IV

ELECTROPHORETIC DATA FOR ALBUMIN AND γ-GLOBULIN

| *Gel* | *Electroosmosis cm* | *Distance of separation cm* | *Corrected length of migration cm* | | *Rate of migration in cm/min × $10^{-2}$* | |
|---|---|---|---|---|---|---|
| | | | *Alb.* | *γ-Glob.* | *Alb.* | *γ-Glob.* |
| Agar gel, 1.25 % | 2.3 | 3.3 | + 3.6 | + 0.3 | + 1.00 | + 0.08 |
| Additions: | | | | | | |
| 3.33 % Sucrose | 1.6 | 3.7 | + 2.8 | — 0.9 | + 0.77 | — 0.25 |
| 3.00 % Dextran L | 1.7 | 3.2 | + 2.7 | — 0.5 | + 0.75 | — 0.14 |
| 0.025 % Carrageenan | 1.4 | 3.7 | + 3.0 | — 0.7 | + 0.83 | — 0.19 |
| 0.100 % Carrageenan | 1.2 | 3.7 | + 2.7 | — 1.0 | + 0.75 | — 0.27 |

+ anodic migration. — cathodic migration.

TABLE V

| *Duration h* | *1.25% Agar gel* | | | | *1.25 % Agar gel + 0.10% Carrageenan* | | | |
|---|---|---|---|---|---|---|---|---|
| | *Electro-osmosis (PVP) cm* | *Corrected rate of migration in cm/min × $10^{-2}$* | | | *Electro-osmosis (PVP) cm* | *Corrected rate of migration in cm/min × $10^{-2}$* | | |
| | | *Alb.* | *$\alpha_2$-Macroglobulin* | *$\gamma$-Globulin* | | *Alb.* | *$\alpha_2$-Macroglobulin* | *$\gamma$-Globulin* |
| 2 | 1.3 | 1.83 | 1.47 | 0.44 | 0.6 | 1.52 | 0.73 | 0.28 |
| 4 | 2.8 | 1.87 | 1.50 | 0.46 | 1.2 | 1.54 | 0.76 | 0.29 |
| 6 | 4.2 | 1.86 | 1.50 | 0.44 | 1.9 | 1.52 | 0.75 | 0.29 |
| Average values | | 1.85 | 1.49 | 0.45 | | 1.53 | 0.75 | 0.29 |

## DISCUSSION

Comparison of the results of diffusion in Table II shows that protein diffusion through 0.666% agar gel is limited; after 48 h the percentage values for serum albumin, $\alpha_2$-macroglobulin and $\gamma$-globulin are 47, 38 and 30, respectively. This slowing down by gel porosities is accentuated by the following additions of carbohydrates. $\alpha_2$-Macroglobulin is slowed down by the addition of Dextran L (mol.wt. 15,000–20,000); the same applies to albumin and to $\gamma$-globulin if Dextran H (mol. wt. 90,000–100,000) or Carrageenan (mol. wt. approx. 300,000) are added to the agar gel. In both these cases gel diffusion of $\alpha_2$-macroglobulin is almost entirely stopped. Carrageenan is a branched chain polysaccharide from seaweed, composed chiefly of D-galactose residues[27, 28]; it increases the viscosity, gel strength and rigidity of the agar gel (see Table III) without chemical reaction (gel turbidity remains low). The increase in the density of the network seems to be independent of the linear D-galactopyranose residues of agar (see Fig. 2).

Thus the change in gel structure is different from the increased $\alpha$-1,6-glucosidic cross-linking of dextran used for the purpose of gel filtration[29]; here branch units, which normally occur only for every 20 glucose residues, are multiplied by chemical reaction. As a result the dextran gel develops from a linear colloid into a three-dimensional network. The agar/Carrageenan combination brings about no molecule sieve either; $\gamma$-globulin diffuses more freely than albumin, a fact which indicates

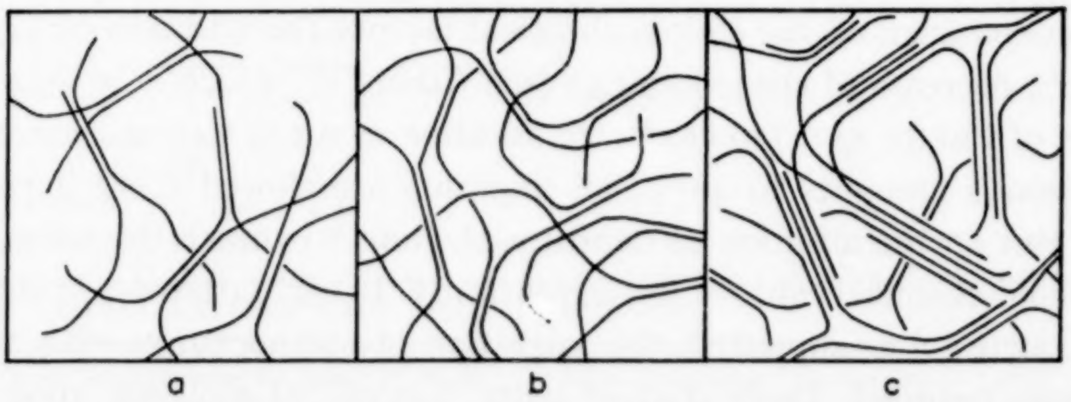

Fig. 2. Schematic representation of network of linear colloids. (a) Agar gel. (b) Agar gel + Carrageenan. (c) Dextran with specially increased cross-linking.

appreciable heteroporosity. The effective pore diameter of a 2 % agar gel is given by TSENG[30] as approx. 30 Å; in this case diffusion would be limited by substances with a molecule radius of more than 2 Å (urea $\nu = 2.7$ Å; sucrose $\nu = 4.4$ Å). If we find that 47 % of the albumin has diffused through the 0.666 % agar gel within 48 h and if we assume that the diffusion rate has been lowered to 50%, then we can deduce that there must be pores with $\nu = 120$ Å[31]. These would also allow $\gamma$-globulin to pass through. As for $\alpha_2$-macroglobulin, no estimates can be given since length and diameter have not been stated, but it can be deduced from the molecular weight, which is twelve times that of albumin, that a considerably wider pore size must exist. Thus it appears that the 0.666 % agar gel is highly heteroporous. The study of percentage distribution of pore sizes is complicated by the fact that the diffusion rate remains equal if the gel membrane contains per cm² a single pore of $\nu = \sqrt{\pi^{-1}}$ cm or $10^4$ pores of $\nu = 10^{-2}. \sqrt{\pi^{-1}}$ cm each. Addition of sucrose generally causes an increase in porosity, whereas addition of Dextran H and of Carrageenan apparently decreases the average pore size; but the results given in Table II convey the impression that large pores fall out earlier than medium-sized pores. As has been stated by MCDONALD[32] for electrophoretic migration in paper, it seems very possible that *different migrants follow different migration paths in gel diffusion*; this would apply particularly in the case of large molecules where intrinsic viscosity[33] and the diffusion constant vary strongly. In our case $[\eta]$ for serumalbumin is [0.034] and for $\gamma$-globulin [0.064–0.066] and $D_{20} \times 10^{-7} = 6.1$ and 3.6, respectively (see Table I). The intrinsic viscosity is a measure of the contribution of the individual protein molecule.

As has been stated already, it was necessary to increase the agar concentration in order to get significant differentiation of electrophoretic migration velocity. Suitable gels must contain 1.25 % of agar; unfortunately, their rigidity is such that measurements of viscosity and gel strength are not possible. Though the measurement of small moduli has become possible lately[34], it is still impossible in the case of gels with a high ratio of shearing stress to strain. We did not try to decrease electroosmosis by purification of the Bacto-agar (WIEME[35]); the important part played by electroosmosis would be less perceptible. To inhibit it altogether by addition of 2 % of gelatinized starch[36] would change all the colloid-chemical factors.The addition of 3.33 % sucrose shows a 30 % decrease of electroosmosis (see Table IV), which is in accordance with the findings of PREER AND TELFER[37]. On addition of 0.1 % Carrageenan the decrease in electroosmosis amounts to 48 %; all migrants are slowed down accordingly (see Table IV). But on the addition of dextrans the effect is much the same, despite the fact that colloid-chemical values differ significantly. In early attempts at electrophoresis with a 1 % agar gel as support[38], the migration of haemocyanin with a mol. wt. of 8,500,000 was recorded. Later studies[8] with "Levan" of mol. wt. 1000 showed unhindered migration in a 2 % agar gel, whereas "Levan" of mol. wt. 1,000,000 was slowed down by 75 %. Our experiments with $\alpha_2$-macroglobulin of mol. wt. 846,000 show a decrease in the rate of migration of 50 % when 0.1 % of Carrageenan is added to the 1.25 % agar gel. With albumin the decrease amounts to 17 % and with $\gamma$-globulin to 35 % (see Table V). Here then *a correlation of molecular size and volume* *with the rate of electrophoretic migration* seems to exist (*cf.* [39,40]). The example of fibrinogen, with a mol. wt. of 400,000 and a molecule thickness the same as that of albumin, which does not move from the starting line even in 0.666 % agar gel, is proof of the importance of the chemical nature of the end groups. Even purified agar specimens contain between 0.3 and 3.7 % sulphur. It is probable that the resulting acidic nature promotes the resolution of normal adult haemoglobin into fractions; if paper is used as support it shows a homogeneous protein. Also in our experiments (Table V) these acidic groups in the agar gel alter the electrokinetic conditions considerably, whereas diffusion of proteins is much more sensitive to alterations in the pore size of the agar gel network. Thus a correlation of systematically altered colloid-chemical gel conditions and electroosmotic flow is readily visible but is not paralleled by changed electrophoretic resolution or separation of protein fractions.

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## SUMMARY

In order to study the possible correlation between protein diffusion, electrophoretic mobility and agar gel structure, the last-mentioned factor has been characterized by means of colloid-chemical methods (viscosity, turbidity, gel strength and rigidity).

Gel diffusion of serum albumin, $\gamma$-globulin and $\alpha_2$-macroglobulin was measured. New limits of diffusion resulted when dextran or Carrageenan were added to the agar gel. Such additions of carbohydrates decreased electroosmosis and tended to slow down the rate of migration. The interdependence is discussed.

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# THERMAL CONDUCTIVITY CELL RESPONSE AND ITS RELATIONSHIP TO QUANTITATIVE GAS CHROMATOGRAPHY

ROBERT L. GROB, DONALD MERCER, THOMAS GRIBBEN
AND JAMES WELLS
*Chemistry Department, Wheeling College, Wheeling, W. Va. (U.S.A.)*

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## INTRODUCTION

Since the appearance of the technique of gas–liquid chromatography many papers have appeared in the literature dealing with the quantitative aspects of the resulting chromatograms[1-10]. There also have been publications dealing with the effects of temperature, pressure and flow rate[11-13] upon gas–liquid chromatographic analyses. In 1958 there was a very elaborate article dealing with the selectivity of liquid substrates for use in gas–liquid chromatography[14]. More recently we have witnessed publications on data presentation[15,16], column efficiency[17], effect of sample size on height of a theoretical plate (HETP) and retention volume[18], and evaluation of detectors for quantitative work[19]. This paper concerns itself with the relationship between thermal conductivity cell response and quantitative gas–liquid chromatography.

For our study we chose a series of alcohols up through hexanol. This project was prompted by the results published previously on hydrocarbons[8].

## THEORETICAL DISCUSSION

Most effort in this field (from the quantitative aspects) has been conducted with an empirical choice of substrates, apparatus and general operating conditions necessary for the separation of specific systems. The main objective we seek is an explanation of why there is a difference in the response of different compounds and in particular, how properties of the various compounds are related to this difference.

It is a known fact that when a thermal conductivity cell is employed as a detector the carrier gas should have a thermal conductivity vastly different from any of the compounds to be determined. Thus, the carrier gas should have a molecular weight extremely large or extremely small (thermal conductivity is inversely related to the square root of the molecular weight of a compound) in order to obtain a significant response from the detector. The low molecular weight of helium and its safety make it ideal for the carrier gas.

If one further assumes that the difference between the thermal conductivity of helium and the compounds under study is large, then one could say the area under the peak is a measure of molar concentration. In so doing the experimenter would introduce sizable errors into his calculations. Most experimenters have found that the peak area is more closely related to weight per cent of a particular component than to the mole per cent. Even so we still may have a sizable error if we correlate peak area to weight per cent. Thus, one must improve his accuracy by calibration.

The big advantage of using peak area (*i.e.* per cent peak area relative to concentration) is that the sample size put onto the column need not be known. Another advantage is that any change in the flow rate during a run will not affect the area of the peak significantly. A change in flow, however, will drastically affect peak height. Therefore, previous calibration by peak height would require extreme care for each sample run. A third advantage is that over fluctuations in the current going through the thermal conductivity cell. This current may change from day to day and cause a change in the sensitivity of the detector. Thus, by using per cent peak area instead of absolute area or peak height calibration you decrease your chances of error.

This use of peak area is the approach we used in this work. All our calculations were made relative to the peak area for a known amount of an alcohol. This is then converted to area per mole.

## APPARATUS AND MATERIALS

*Fractometer*: Perkin-Elmer Vapor Fractometer, Model 154C, manufactured by the Perkin-Elmer Corporation, Norwalk, Conn.

*Recorder*: Leeds and Northrup Speedomax Type G Recorder, 10 mV.

*Balance*: Christian Becker Chainomatic Magnetically Damped Balance, Model AB-2.

*Column*: ¼ inch O.D. copper tubing, 10 ft. in length.

*Column support*: Fisher Columnpak, 30–60 mesh, purchased from the Fisher Scientific Company, Pittsburgh, Pa.

*Liquid substrate*: Eastman technical tritolyl phosphate purchased from Eastman Kodak Company, Rochester, New York.

*Carrier gas*: Helium.

*Alcohols*: High purity alcohols purchased from Eastman Kodak Company, Rochester, New York or Fisher Scientific Company, Pittsburgh, Pa.

## METHOD

Each alcohol investigated was blended with a known weight of internal standard. The internal standard used for this investigation was normal propyl alcohol. Each blend was run ten times and the operation repeated on a second similar blend. 0.05 ml samples were used for all the runs.

After separation through the 10 ft. column of tritolyl phosphate the area for each alcohol was determined by two methods. First by integral calculation, *i.e.* multiplying the peak height by one-half the band width in centimeters; second by cutting out the peak and weighing it on an analytical balance.

For the second method the uniformity of the paper was determined by cutting out and weighing known areas from different portions of the chart roll. Error due to non-uniformity of the paper and the cutting and weighing processes was found to be less than 1 %.

The area of the peak was then determined on the basis if 1 mole of the alcohol was passed through the column. This area per mole value was in turn used to calculate the relative response per mole setting the internal standard to a value of unity.

## EXPERIMENTAL RESULTS

To measure the detector response of a particular alcohol on either a mole or weight basis and compare this response value to other alcohols, it is necessary to introduce on to the partition column a precisely known amount of each alcohol. Also the sensitivity of the detector, the flow rate and the temperature must be the same for each determination. Maintaining these conditions the same from run to run and day to day is not easy, thus each alcohol is blended with an internal standard. In this manner the only requirement is that the operating conditions remain constant for the duration of a run. Thus the ratio of the area of the internal standard to that of a particular alcohol in question is independent of sample size and the volume and weight of sample need not be known.

Table I shows the alcohols investigated and the average value of the response per mole (R.P.M.) for each one. The response per mole was calculated on both a weight

TABLE I

RELATIVE RESPONSE VALUES PER MOLE OF ALCOHOL

| *Alcohol* | *R.P.M.** *by weight* | *R.P.M.** *by integral* |
|---|---|---|
| Methanol | 0.45 | 0.45 |
| Ethanol | 0.74 | 0.74 |
| *n*-Propanol | 1.00 | 1.00 |
| Isopropanol | 1.04 | 1.04 |
| *n*-Butanol | 1.27 | 1.27 |
| *tert.*-Butanol | 1.27 | 1.27 |
| Isobutanol | 1.86 | 1.86 |
| *sec.*-Butanol | 1.20 | 1.20 |
| *n*-Pentanol | 1.46 | 1.46 |
| Isopentanol | 1.56 | 1.56 |
| *tert.*-Pentanol | 1.54 | 1.54 |
| 3-Pentanol | 1.38 | 1.38 |
| 2-Methyl-1-butanol | 1.32 | 1.32 |
| 3-Methyl-2-butanol | 1.65 | 1.65 |
| 2-Hexanol | 1.59 | 1.59 |
| 2-Methyl-2-pentanol | 1.59 | 1.59 |

* Response per mole (all values calculated relative to *n*-propanol; *n*-propanol = 1.00). Conditions: 130°, 25 psi helium; 10 ft. column of tritolyl phosphate on Columnpak 40:60.

basis (cutting out and weighing the peaks on an analytical balance) and an integral basis (product of peak height and one-half the band width). The same value for response per mole was obtained by both methods. From this we concluded that it makes little difference which method was used for determining the area under the peak.

Fig. 1 shows a plot of these response values, for the normal alcohols, versus the square root of the molecular weight. A linear relationship was found. The values for the isomeric forms of the alcohols could not be plotted on a similar graph because:

1. Their values did not follow any linear relationship.
2. We did not have enough isomers to justify such a plot.

We then plotted our response values against certain properties of the alcohols to see if any correlation could be found other than square root of the molecular weight. Fig. 2 shows a plot of these response values versus molecular volume. Here too a linear relationship was found. This linear relationship could be expected since molecular volume is related to molecular weight and density of a compound. Looking at the response values for the normal alcohols we find that as we add a carbon atom to the chain from methanol to butanol the increase in R.P.M. is fairly constant. But when we increase to five carbons (*n*-pentanol) our increment is decreased. If we carry this further to six carbons (2-hexanol) we again find a decrease in the increment. Table II shows the change in R.P.M. from methanol through 2-hexanol. From this we could postulate that as we increase the number of carbons on the chain we would eventually reach a point where very little change would occur in our R.P.M. values. If this were true then with higher molecular weights the area under the peak for all alcohols would essentially be the same and we could obtain accurate analyses by assuming peak area was directly related to weight per cent. Our inability to obtain high purity alcohols in this molecular weight range prevented any further investigation in this area.

TABLE II

CHANGE IN R.P.M. VALUES PER CARBON ATOM

| *Alcohol* | *R.P.M.* | *Change in R.P.M.* |
|---|---|---|
| Methanol | 0.45 | |
| Ethanol | 0.74 | 0.29 |
| Propanol | 1.00 | 0.26 |
| Butanol | 1.27 | 0.27 |
| Pentanol | 1.46 | 0.19 |
| 2-Hexanol | 1.59 | 0.13 |

Fig. 3 shows that we also obtain a reasonable relationship between boiling point and response per mole (again only a plot of the normal alcohols is shown because they alone follow a pattern).

Figs. 4 and 5 show a similar correlation for vapor pressure at 20° and 130°, respectively.

Once having obtained these values for the response per mole of our alcohols we then proceeded to test them under actual experimental conditions. Blends made up of four and five alcohols were investigated. These blends were run under the same conditions as our initial two-component blends, *i.e.*, 130° temperature, 25 psi pressure of helium and 0.05 ml sample size. Table III shows the results of these runs. Each blend was run six times and the average values used.

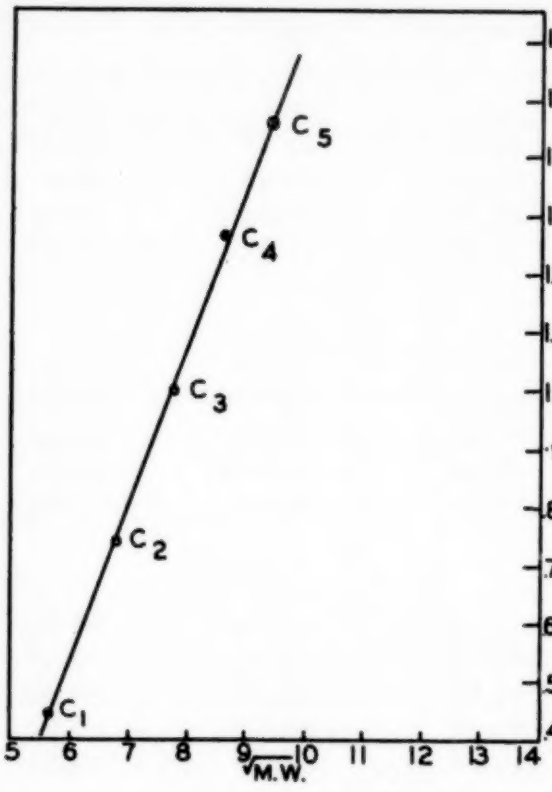

Fig. 1.

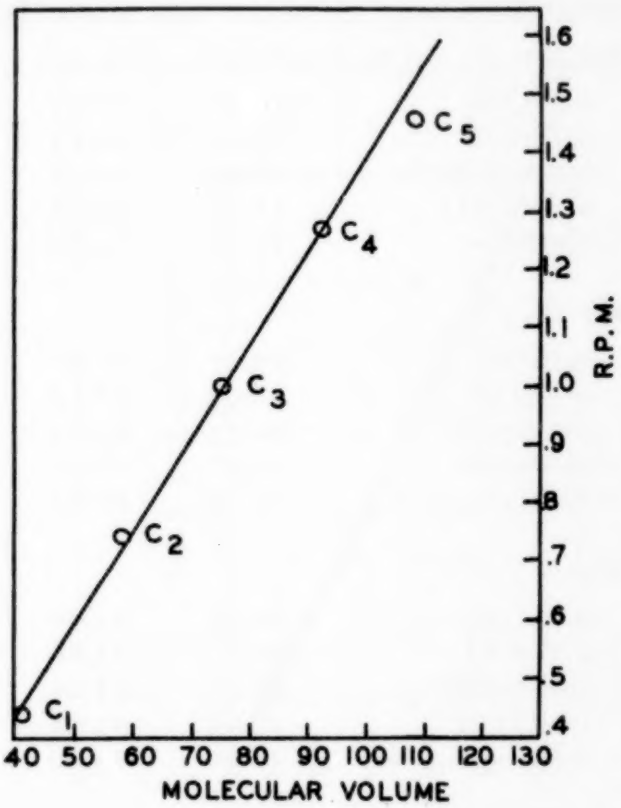

Fig. 2.

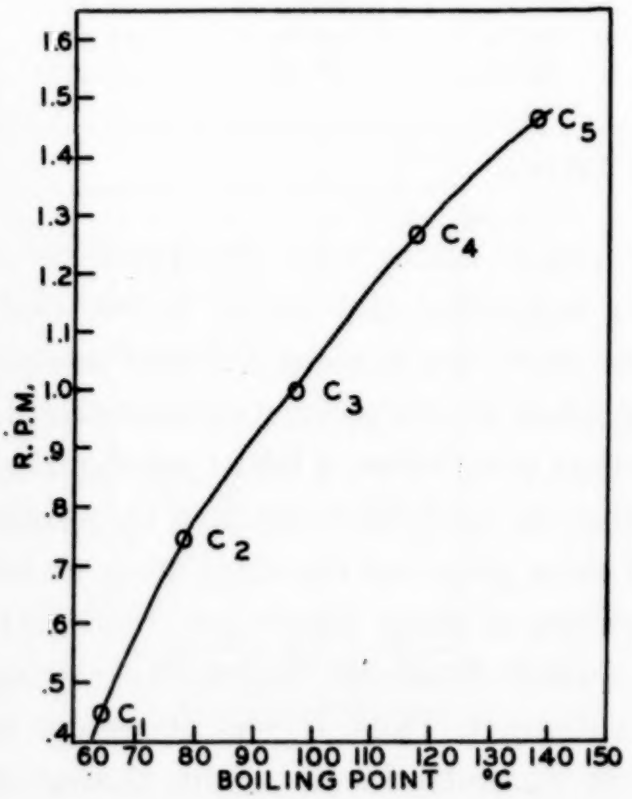

Fig. 3.

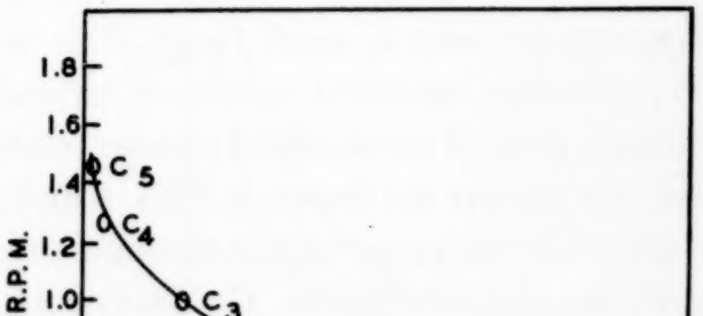

Fig. 4.

If one assumed that area was equal to mole per cent, errors as high as 26.3 % were encountered; when area assumed equal to weight per cent, errors as high as 10.7 % were encountered. If the areas were corrected by the response values the error was less than 1.0 %.

TABLE III

ANALYSIS OF SYNTHETIC BLENDS

| *Blend alcohols* | *True wt. %* | *True mole %* | *Observed area %* | | *Calculated* %* | |
|---|---|---|---|---|---|---|
| | | | *by weight* | *by integral* | *by weight* | *by integral* |
| *101-D* | | | | | | |
| *n*-PrOH | 16.71 | 17.30 | 14.21 | 14.02 | 16.86 | 16.67 |
| *n*-BuOH | 24.11 | 20.19 | 25.79 | 25.62 | 24.18 | 24.03 |
| *iso*-AmOH | 20.39 | 14.42 | 27.00 | 27.34 | 20.48 | 20.31 |
| *n*-AmOH | 21.77 | 15.38 | 26.64 | 26.66 | 21.63 | 21.69 |
| MeOH | 17.02 | 32.69 | 6.36 | 6.36 | 16.95 | 16.98 |
| *102-D* | | | | | | |
| *n*-PrOH | 19.47 | 21.90 | 16.07 | 16.10 | 19.54 | 19.41 |
| *n*-BuOH | 18.97 | 17.14 | 19.82 | 19.85 | 19.06 | 18.92 |
| *iso*-AmOH | 20.53 | 15.23 | 26.46 | 26.47 | 20.57 | 20.42 |
| *n*-AmOH | 21.07 | 16.19 | 25.40 | 25.38 | 21.16 | 20.97 |
| EtOH | 19.95 | 29.52 | 12.24 | 12.20 | 20.06 | 19.84 |
| *103-D* | | | | | | |
| *n*-PrOH | 19.91 | 25.00 | 14.12 | 14.09 | 20.03 | 19.86 |
| 2-HxOH | 19.64 | 13.88 | 22.05 | 22.03 | 19.74 | 19.58 |
| *iso*-BuOH | 20.70 | 21.29 | 27.30 | 29.87 | 20.78 | 20.63 |
| *iso*-PrOH | 20.93 | 25.92 | 15.39 | 15.42 | 20.97 | 20.87 |
| 2-Me-2-AmOH | 18.80 | 13.88 | 21.15 | 21.16 | 18.86 | 18.74 |
| *104-D* | | | | | | |
| *n*-PrOH | 28.09 | 35.29 | 22.76 | 22.78 | 28.14 | 28.01 |
| *tert.*-BuOH | 20.58 | 21.17 | 21.27 | 21.25 | 20.64 | 20.52 |
| 3-AmOH | 23.79 | 20.00 | 26.62 | 26.60 | 23.85 | 23.70 |
| 2-Me-1-BuOH | 27.53 | 23.52 | 29.34 | 29.37 | 27.58 | 27.47 |

* Percentages calculated using the R.P.M. values in Table I.

It was previously shown[8] that relative response values were independent of temperature, over a small range. This was later supported and shown to be true[6]. We, therefore, analyzed several of our multi-component blends using different sample volumes to see if we obtained a linear relationship when area is plotted against sample size. The results are shown in Figs. 6 and 7. As these plots show, a linear relationship is held over the range of 5 μl through 50 μl whether we calculated the area by weight or by integral calculation. The areas depicted in these plots are the total areas under all the peaks of a single blend. For the composition of these blends see Table III.

In some of our runs it was necessary to switch from one sensitivity setting (attenuation) to another to have all the peaks distinct. Thus, it was necessary to check to see whether or not any appreciable error was introduced by this change in sensitivity. Fig. 8 shows that we obtained a fairly good relationship between sensitivity and total area under the peaks. The maximum relative error due to change in attenuation, in any one run was less than 1 %.

Our last point to investigate was whether a linear relationship was held when the weight per cent of an alcohol was varied in a blend. This was done by means of blends made up on a weight ratio basis. A series of eleven blends were made using methanol and *n*-propanol. The blends were made up by adding drops of each alcohol to a vial. The weight of each alcohol was found by means of an analytical balance. The total number of drops of solution was the same in each blend, just the ratio of methanol to propanol was varied. The first blend contained 10 drops of *n*-propanol and 0 drops of methanol. The drops of methanol were increased while the drops of propanol were decreased, keeping the total always 10 drops, until the eleventh blend which contained 10 drops of methanol and 0 drops of *n*-propanol.

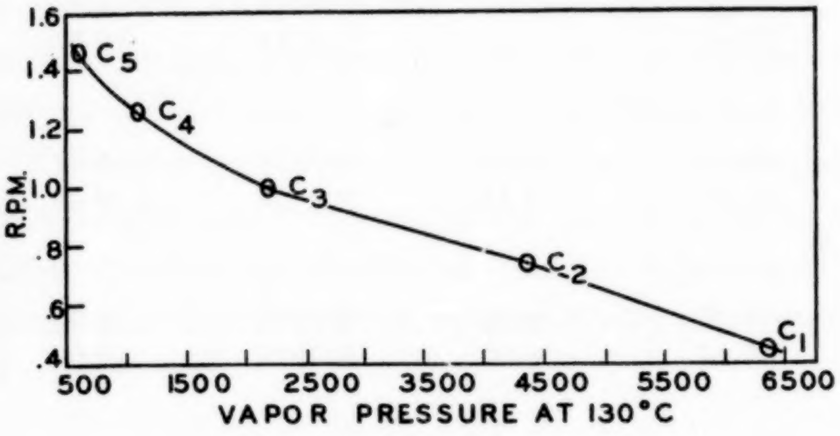

Fig. 5.

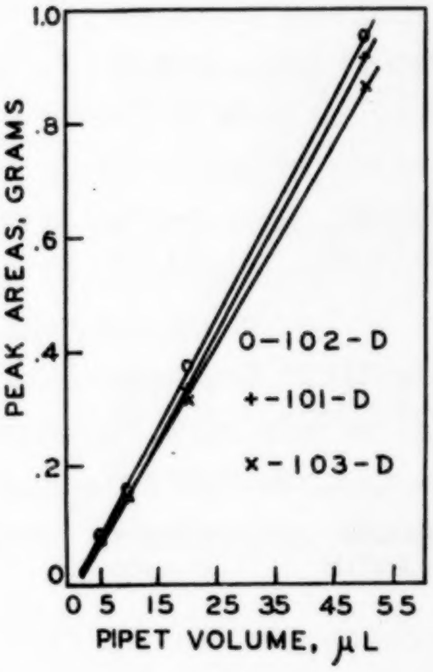

Fig. 6.

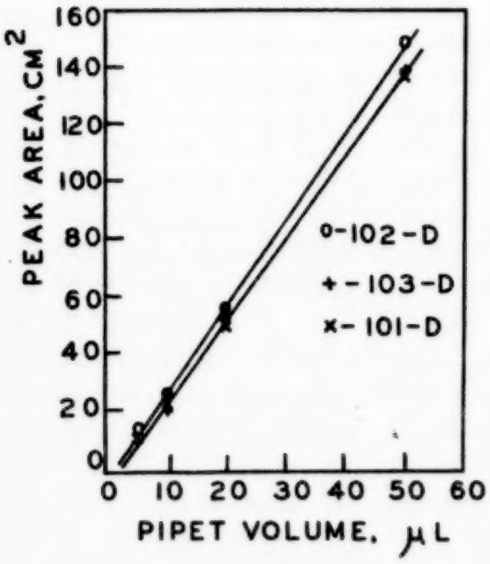

Fig. 7.

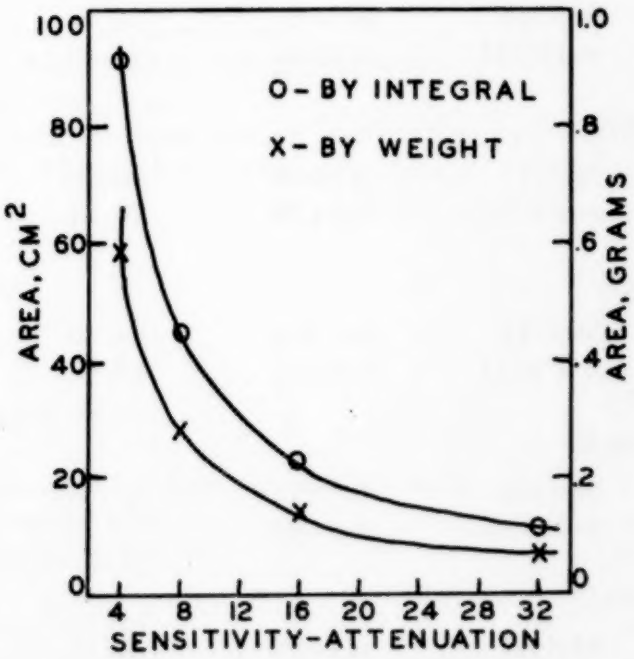

Fig. 8.

Table IV shows the weight of each alcohol, the peak height, peak area, mole per cent and weight per cent of each alcohol for the eleven blends.

All blends were run at a sensitivity of 32 and a 0.05 ml sample size employed for each run. From the results one sees that the response values hold over a wide concentration range.

## TABLE IV

### EFFECT OF CONCENTRATION ON PEAK AREA

| *Blend* | *Weight in blend* g | *Weight per cent* | *Mole per cent* | *Peak height* cm | *Area by weight* g | *Area by integral* cm$^2$ |
|---|---|---|---|---|---|---|
| *1E* | | | | | | |
| *n*-PrOH | 0.1705 | 100.0 | 100.0 | 7.59 | 0.0858 | 13.05 |
| *2E* | | | | | | |
| MeOH | 0.0145 | 8.6 | 14.95 | 1.34 | 0.0075 | 1.11 |
| *n*-PrOH | 0.1541 | 91.40 | 85.05 | 6.93 | 0.0800 | 10.79 |
| *3E* | | | | | | |
| MeOH | 0.0285 | 16.99 | 27.59 | 2.94 | 0.0151 | 2.22 |
| *n*-PrOH | 0.1392 | 83.01 | 72.41 | 6.33 | 0.0744 | 10.85 |
| *4E* | | | | | | |
| MeOH | 0.0433 | 29.68 | 44.26 | 5.23 | 0.0274 | 3.97 |
| *n*-PrOH | 0.1026 | 70.32 | 55.74 | 5.44 | 0.0651 | 9.40 |
| *5E* | | | | | | |
| MeOH | 0.0563 | 36.49 | 51.78 | 6.39 | 0.0345 | 4.94 |
| *n*-PrOH | 0.0980 | 63.51 | 48.22 | 4.96 | 0.0602 | 8.60 |
| *6E* | | | | | | |
| MeOH | 0.0672 | 46.60 | 62.02 | 7.98 | 0.0448 | 6.41 |
| *n*-PrOH | 0.0770 | 53.40 | 37.98 | 4.18 | 0.0511 | 7.35 |
| *7E* | | | | | | |
| MeOH | 0.0952 | 58.73 | 72.79 | 9.62 | 0.0583 | 8.35 |
| *n*-PrOH | 0.0669 | 41.27 | 27.21 | 3.37 | 0.0412 | 5.87 |
| *8E* | | | | | | |
| MeOH | 0.1108 | 69.86 | 81.37 | 11.22 | 0.0724 | 10.33 |
| *n*-PrOH | 0.0478 | 30.14 | 18.63 | 2.41 | 0.0313 | 4.45 |
| *9E* | | | | | | |
| MeOH | 0.1425 | 81.48 | 89.34 | 12.50 | 0.0856 | 12.35 |
| *n*-PrOH | 0.0324 | 18.52 | 10.66 | 1.45 | 0.0194 | 2.81 |
| *10E* | | | | | | |
| MeOH | 0.1652 | 92.24 | 95.72 | 13.73 | 0.0973 | 14.24 |
| *n*-PrOH | 0.0139 | 7.76 | 4.28 | 0.55 | 0.0080 | 1.20 |
| *11E* | | | | | | |
| MeOH | 0.1778 | 100.0 | 100.0 | 14.78 | 0.1047 | 15.90 |

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## SUMMARY

By analyzing two component blends of alcohols (one being an internal standard) we were able to compute response values, on a mole basis, for various alcohols. These computed values were then used to calculate weight per cents of alcohols in multi-component blends. The blends were analyzed by gas chromatography using a thermal conductivity cell as the detector. As a result of this investigation we found that the area under the peak of a chromatogram is a measure of the weight per cent of an individual component rather than the mole per cent. If the per cent area was used directly as a measure of weight per cent errors as high as 10.7% were encountered. The use of our computed response values cut the error to less than 1.0 %

Correlation between concentration and peak area, sensitivity and peak area, peak height and concentration, and peak area and concentration were also investigated. In all cases a low error relationship was found. This indicated that our response values were affected little by sensitivity (attenuation) changes, concentration changes or sample size.

We hope later to be able to correlate all these data with specific properties of various compounds.

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# A MULTICOLLECTOR: A FRACTION COLLECTOR FOR THE SIMULTANEOUS COLLECTION OF FRACTIONS FROM A NUMBER OF CHROMATOGRAPHIC COLUMNS

PER VESTERGAARD

*Research Facility, Rockland State Hospital, Orangeburg, N.Y. (U.S.A.)*

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In a longitudinal study of steroid metabolism in schizophrenic patients we were faced with the problem of having to run large numbers of column chromatographic assays per day. It was not economically feasible for us to buy the number of individual commercial units necessary to cover our need, nor did we have the space to accommodate them. We decided therefore to build a special unit for multiple simultaneous collections. To suit its purpose the collector had to be simple of design for ease in construction, give trouble-free service with little maintenance, and be far less expensive than commercial units that would give the same capacity.

Fig. 1 shows the collector we built to solve our problem. The principle of construction is simple. Test-tube carriers mounted on wheels ride on rails mounted on a slight slope. A latch activated by a recycling timer allows the carriers to slide down the slope for a distance equal to the distance between the test tubes. The time interval is set by adjusting one of the two recycling timers.

## DETAILS OF CONSTRUCTION

### *The support stand for the rails* (Fig. 1)

This stand can be built, as in our earlier models, with a plywood top on a 2 in. × 4 in. lumber frame as support for the rails.

However, a more flexible system is obtained if slotted steel angle framing (Equipto Angle, Aurora, Ill.) is used. This material is inexpensive, easily cut and can be bolted together to suit individual needs.

A sloping frame support for the rails is made by bolting two angles in a parallel position to crossbars cut from the same material. The rails (1/4 in. angle aluminum) are screwed directly to the angles in the frame with uniform spacing maintained for the entire distance.

The bottom part of the rails are curved upwards to a horizontal position at the end so that the cars will not slam against the stop located near the end of the rails.

This sloping frame is supported by 4 vertical pieces of angle iron and these are connected at the bottom to a horizontally placed bottom frame similar to the

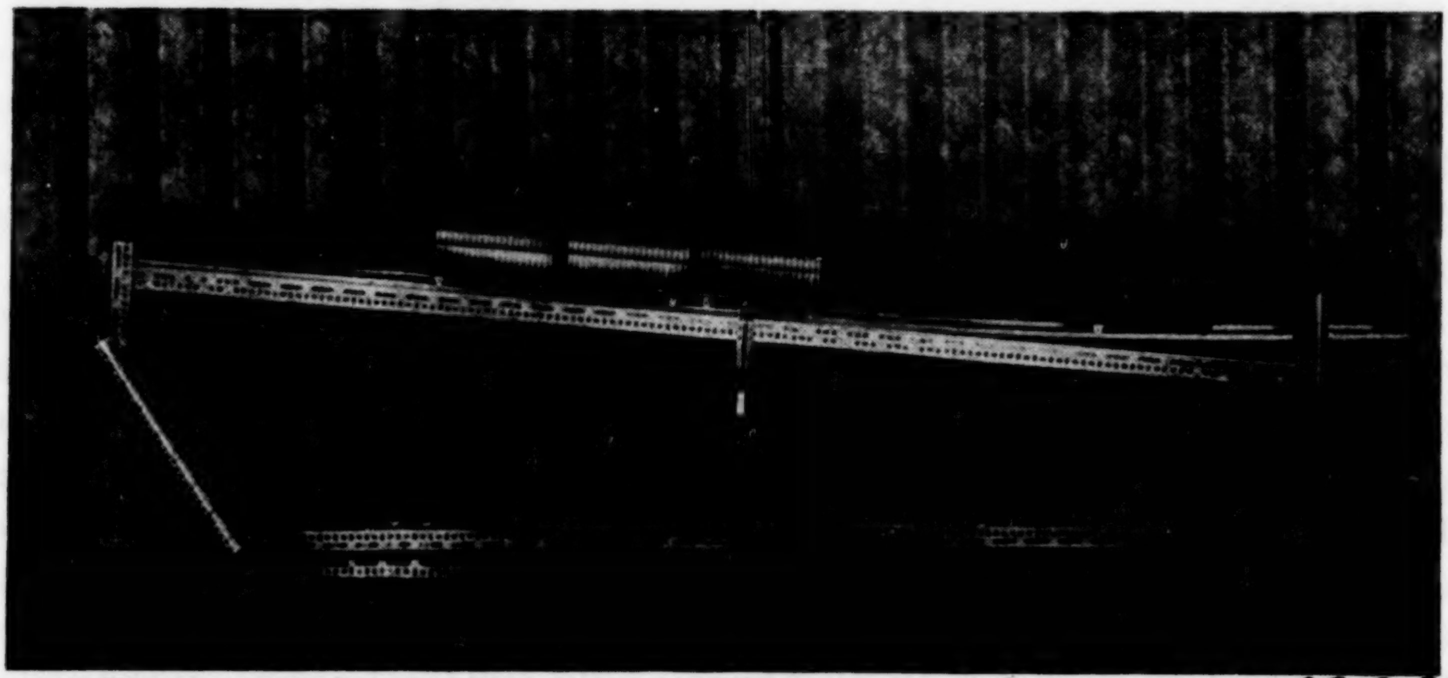

Fig. 1. A multicollector for the simultaneous collection of fractions from six chromatographic columns.

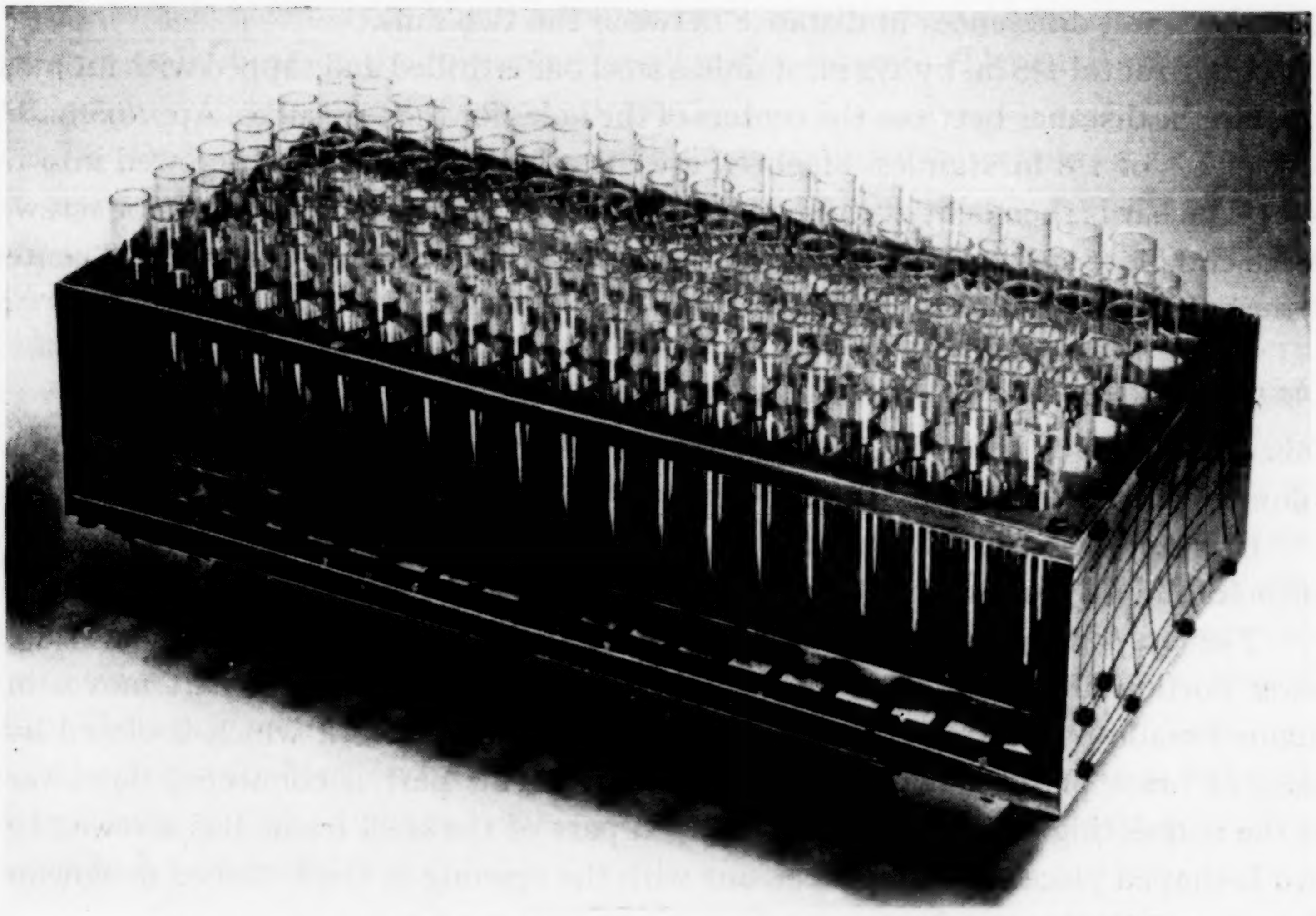

Fig. 2. A test-tube carrier with 6 rows of test tubes and 20 tubes per row.

sloping frame. The bottom frame is placed on floor or table top. A couple of crossbars give stability to the lower frame but are unnecessary if two or three of these units are combined.

*The test tube carriers* (Fig. 2)

These are rectangular aluminum plate boxes with open sides and are mounted on wheels. Small stainless steel pins positioned on a rectangular bar on the side engage the solenoid activated latch on the fraction collector.

The tube carriers are made from 1/62 in. aluminum plate screwed to a frame of 1/4 in × 1/4 in. aluminum bar. Our standard model has three horizontal rectangular aluminum plates and two vertical rectangular plates. Six rows of 20 holes per row are drilled in two of the horizontal plates. The third plate serves as a bottom. The bottom plate and one of the drilled plates have been screwed to opposite surfaces of a rectangular frame made of 1/4 in. × 1/4 in. aluminum bar and the other drilled plate has been screwed to the top surface of a similar rectangular frame. They have then been assembled to make an open box with the two smaller rectangular plates as end plates.

The wheels for the carriers were turned on a lathe from aluminum rod. The wheels on the side of the carrier where the pins are located are grooved slightly wider than the thickness of the rail to insure that the carrier is kept at constant distance from the latch. The wheels on the other side are rather wide ungrooved cylinders to allow for small differences in distance between the two rails.

A horizontal 1/8 in. by 1/2 in. stainless steel bar is drilled and tapped with intervals equal to the distance between the centers of the holes for the test tubes. Approximately 1-in. pieces of 1/8 in. stainless steel rod are threaded at one end and screwed into the holes. The bar is then bent at right angles at the ends and, with a few washers, screwed to the end plate and positioned in such a way that the pins are in line with the centers of the test tube openings.

*The release/engage mechanism* (Fig. 3)

This device serves to position a new row of test tubes under the chromatographic columns when activated by the timer.

It is comprised of a top portion, a connecting rod with an adjustable spring, a solenoid and an aluminum mounting plate.

*The top portion* consists of a frame with a U-shaped upper portion and a straight lower portion and is milled from a single piece of steel. The lower part moves in a channel made by cutting an oversized groove in a brass block which is closed by a piece of brass plate fastened across the groove. This part is connected downwards to the connecting rod. The upper U-shaped part of the steel frame has screwed to it two L-shaped pieces of spring steel, one with the opening in the L turned downwards, the other with the opening upwards.

*The connecting rod* is a piece of 1/8 in. steel rod connected upward to the head portion, and downward to the solenoid. It has an adjustable spring held between two washers. One washer is held in a fixed position by an L-shaped piece of aluminum

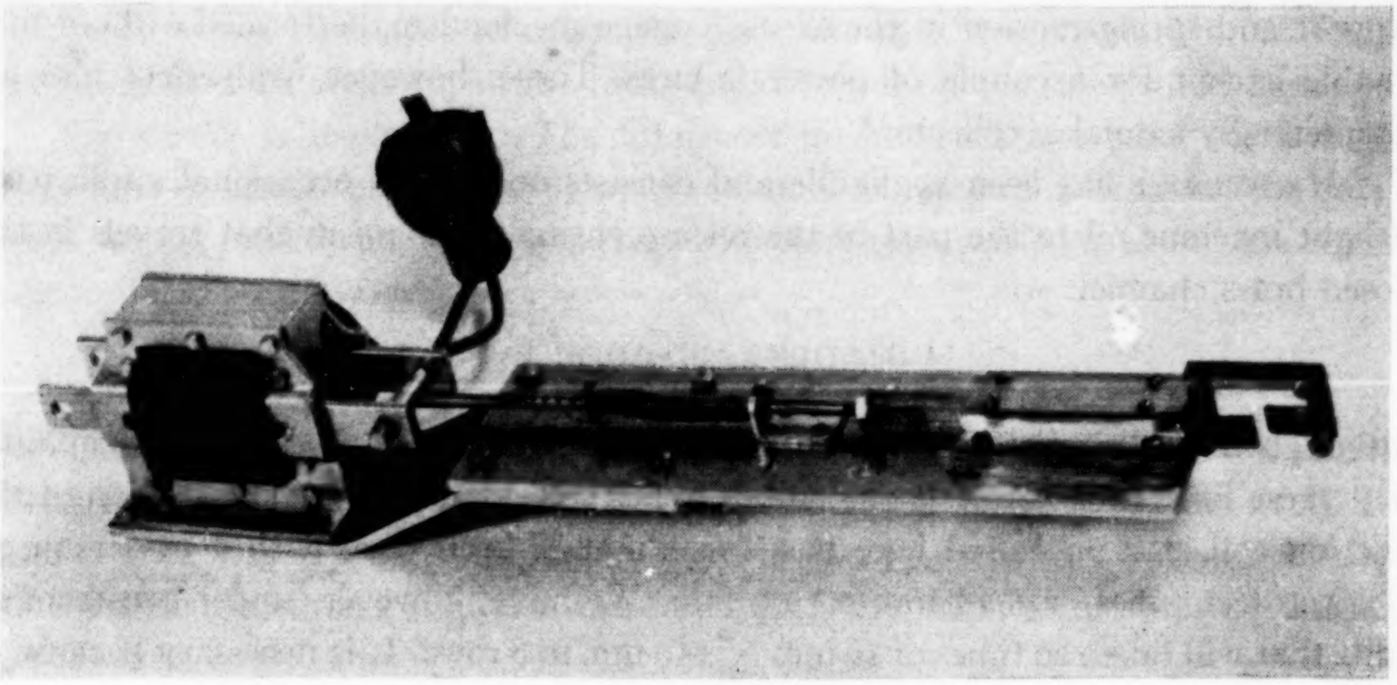

Fig. 3. The release/engage mechanism.

plate screwed to the mounting plate, the other washer can be moved upwards or downwards by moving a small cotter pin for which small holes have been drilled in the connecting rod.

*The solenoid* (2000 Size C-1, Dormeyer Industries, Chicago 41) is of the push-pull type, mounted in the push position.

The whole assembly is aligned and screwed on an 1/8 in. thick aluminum plate that has to be bent at the lower end to align the solenoid. The total mechanism is then screwed to the fraction-collector frame.

*The timer* is used to activate the release/engage mechanism after a set interval. We have chosen, from several commercially available units, a tandem automatic recycling timer (Industrial Timer Corporation, Newark, New Jersey) which employs two interchangeable timers. One, a 0-6 sec timer, is used to activate the solenoid and is set for approximately 3/5 of a second.The other timer is used for timing the interval between activations. In the model used, interchangeable plug-in timers covering a wide range are available so that one can choose a timer suited for any specific purpose. A timer with a 60 min maximum interval between activations of the second timer and the solenoid in the release/engage mechanism will probably cover most applications.

In use the solenoid is connected with the line voltage over the normally open contacts of timer 2. Timer 2 is activated together with the solenoid for 3/5 of a second after expiration of the interval set on timer 1 and timer 1 will start recycling immediately. This cycle continues until the unit is disconnected from the line voltage.

The firm estimates, on the basis of the performance of similar units in industrial applications, that the average trouble-free life of the timer in its present application should be 4 to 5 years.

## PERFORMANCE

Several collectors of this type have been in almost continuous use in our laboratories over the last year and have, after initial adjustments of the proper slope of the rail support and spring tension in the release/engage mechanism, performed without any trouble except for a couple of power failures. These, however, will affect also all commercially available collectors.

*Maintenance* has been negligible and consists only of an occasional application of light machine oil to the part of the release/engage mechanism that travels in the closed brass channel.

## LIMITATIONS AND ADVANTAGES

Although the collector has in its present version some limitations, it is our opinion that these limitations are relative only. Capacity is one problem. The version of the fraction collector presented here has, for example, been used with a maximum of 6 × 100 test tubes, size 13 mm × 100 mm. We have, however, under construction units that will take 120 tubes of 16 mm × 150 mm in 8 rows. It is necessary because of the increased load to use a heavier solenoid and corresponding spring, and also to use heavier spring steel. It will be possible to run, because of the higher electrical rating of the solenoid, only 2 units from the same timer.

The length of the units will present a problem in some laboratories. The length of a 120 tube 13 mm diameter collector is 12½ feet. However, three such units can be placed alongside of each other on a tabletop or on a corresponding area on the floor, and give a capacity of 18 columns. The smallest commercially available unit has a width of 15 inches and 18 such units would take up 50% more space.

We have at present one unit on the floor under two laboratory tables that are placed about 3 feet from each other. The space between the tables is then used for the setting up of the chromatographic columns and the area on the tabletops is available for other work. These narrow types of collector can also be set up in (well ventilated) hallways where one often finds otherwise wasted space. Another factor to consider is that these units can be partially disassembled in about 10 min by unbolting the vertical supports from the horizontal and sloping part of the collector and the components can then be stored on a shelf.

The most important advantage of this collector design is that it allows many columns to be run at the same time. This will be of greatest value in cases of serial analyses where long runs are necessary to get good analytical results and where the number of columns that can be run will be the limiting factor in the analysis. In many other cases considerable time per analysis will be saved since the analyses can be set up on an "assembly" line basis with a corresponding cut in time spent per analysis.

The collector is particularly useful when used in connection with the technique described elsewhere in this issue[1] for simultaneous gradient elution chromatography on a number of columns.

It is furthermore very flexible in design. Length and width can be adapted to the problem at hand and the test tube carriers can be built to take test tubes of many different sizes, and if needed, flasks or beakers.

The test tube carriers can be put directly in a large vacuum oven for evaporation of organic solvents. The carriers in the present version have permanently attached wheels. It would be simple to make the test tube rack detachable from the wheel portion so that different type tube racks could be put on the same wheel base.

The device is inexpensive. The total cost in materials and labor for a duplex unit that can handle 12 chromatographic columns is somewhat less than the cost of one of lowest priced commercial fraction collectors that can be used for one column only.

## ACKNOWLEDGEMENTS

The author expresses his appreciation to his colleague, JOHN BLAIR, for much valuable advice and to L. G. COLLYER, who did the mechanical construction involved. This piece of work was supported in part by a grant (M 1953) from the National Institute of Mental Health of the Public Health Service of the Department of Health, Education and Welfare.

## SUMMARY

A fraction collector for the simultaneous collection of fractions from a number of chromatographic columns is described. The collector is simple in design, easy to construct and is inexpensive to build. Limitations and advantages of the apparatus are discussed.

## REFERENCE

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# TECHNIQUE FOR SIMULTANEOUS MULTIPLE-COLUMN GRADIENT ELUTION CHROMATOGRAPHY

PER VESTERGAARD

*Research Facility, Rockland State Hospital, Orangeburg, N.Y. (U.S.A.)*

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Several methods for gradient elution chromatography have been proposed since ALM *et al.*[1] published the first detailed study of this technique. They have all been worked out for use with a single column.

The technique presented in this article has been primarily developed as a method for running multiple simultaneous chromatograms using the gradient elution method. It does, however, also offer a system for single column gradient elution chromatography that combines relative simplicity with flexibility. The fact that the gradient can be changed at will in a predetermined way at any time during the run by simply turning a screw control should prove especially useful.

The first problem we ran into in developing the technique was the unavailability of an accurate device that could deliver small volumes of eluant to a developer and at the same time could be varied continuously and easily in a calculated way.

The dropping funnel described by LAKSHMANAN AND LIEBERMAN[2] in their gradient elution method was tried but not found accurate enough for very small volumes, and the adjustment to a given volume was a long and cumbersome procedure.

A variable speed syringe drive was developed to solve these problems.

## VARIABLE SPEED SYRINGE DRIVE

This device consists (Fig. 1) of a Krogh-Keyes syringe pipette arranged so that it can slide freely on two metal bars, a continuously variable speed changer with a screw control, and a synchronous motor, all mounted on a 1/4-in. thick aluminum plate.

A Krogh-Keyes syringe pipette (MacAlaster Bicknell No. 34197) of appropriate size is fitted with four 2-in. long 1/8 in. × 3/8 in. aluminum bars. They are placed so that they are parallel with the part of the Krogh-Keyes syringe that is mounted around the collar of the syringe barrel and are attached to the four corners of the metal frame of the syringe pipette. The grooves in these vertical aluminum bars slide over two horizontal stainless steel rods mounted on two small aluminum posts screwed into the 1/4 in. aluminum base. With this arrangement the syringe can move freely back and forth in a horizontal position. A circular piece of stainless steel plate 1/32 in. thick is glued to the end of the syringe piston to ensure a flat surface.

The threaded rod in the Krogh-Keyes syringe is connected to the variable speed

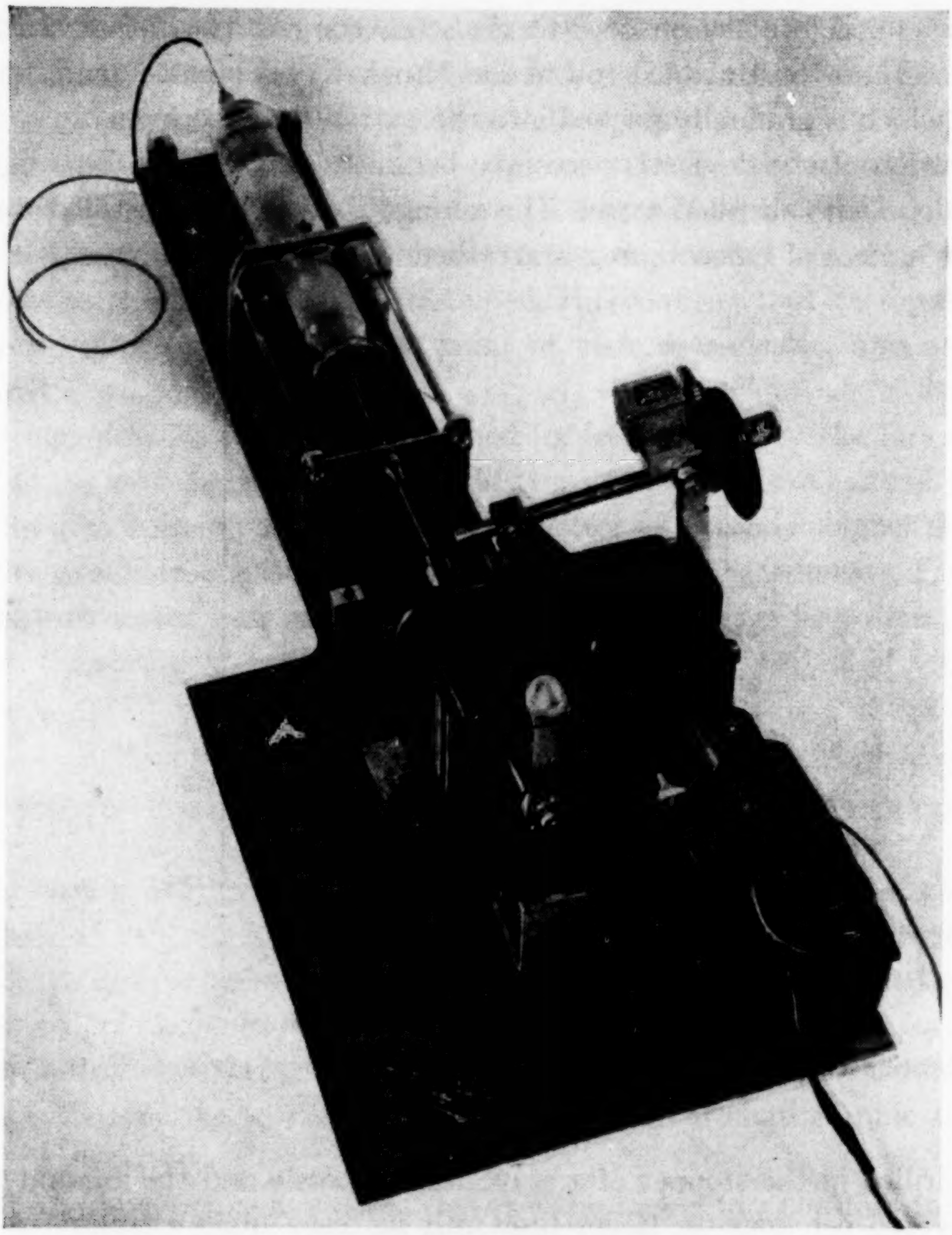

Fig. 1. The variable speed syringe drive with Krogh-Keyes syringe pipette, variable speed changer with screw control and counter and synchronous motor.

changer (Revco, Minneapolis, Model 14R) with a small cylindrical brass connector.

A counter is mounted on the screw control of the variable speed changer. A 1-in. diameter 48 pitch brass spur gear is fastened on the axle of the counter and a similar gear is put on the threaded rod which drives the screw control. One turn of the screw control registers as one turn on the counter and the screw control has a range of either 0 to 50 or 0 to 500 if subdivisions are used. This corresponds to a continuous variable speed reduction of the input speed from the motor of 1/4 of motor speed to zero.

The variable speed changer is connected to a synchronous motor. The speed selected for this motor is 1 r.p.m. for our application. If delivery greater than approximately 10 ml/h is necessary, a faster motor must be used.

The device operates in such a way that the rotating movement from the synchronous motor shaft is transferred to the output shaft of the variable speed drive with whatever speed reduction is set on the screw control. The output shaft from the speed drive drives the threaded rod in the Krogh-Keyes pipette against the end of the piston which is gradually pushed into the barrel of the syringe.

Calibration of the device is necessary before it can be used. This is most conveniently done with distilled water. The syringe is filled with distilled water and a needle with a piece of teflon tubing is attached. A hole slightly larger than the teflon tubing is drilled in the stopper of a polyethylene bottle and the amount of distilled water delivered per hour (or larger time unit for very small amounts) is measured. Fig. 2 shows such a calibration chart for a 50 ml and a 10 ml syringe. Syringes with interchangeable pistons like the multifit type are preferable, since one calibration can then be used for a number of syringes with the same capacity.

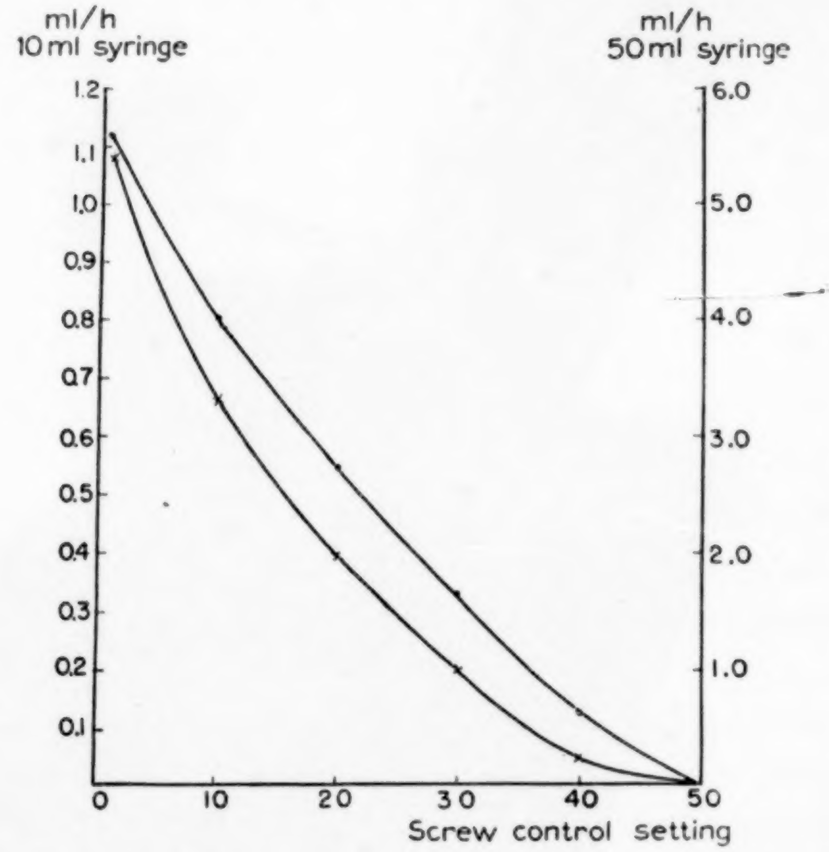

Fig. 2. Calibration chart for 50 ml (×—×—×) and 10 ml (o—o—o) syringes in the variable speed syringe drive.

The accuracy and reproducibility of the syringe drive was tested by running 10 calibrations of both a 50 ml and a 10 ml syringe at a slow setting of the screw control (40.0) and again at a fast delivery rate (screw control set at 10.0). The means and

TABLE I

REPRODUCIBILITY OF SYRINGE DRIVE

Output per hour given as mean value with standard deviation for 10 determinations at a slow and fast delivery rate.

| | *Screw control set at 10.0* | | *Screw control set at 40.0* | |
|---|---|---|---|---|
| | *Mean ml/h* | *Standard deviation* | *Mean ml/h* | *Standard deviation* |
| 50 ml syringe | 3.327 | 0.027 | 0.219 | 0.0035 |
| 10 ml syringe | 0.807 | 0.0075 | 0.132 | 0.0025 |

standard deviations have been calculated for both these settings. Table I shows the degree of accuracy obtained, with a standard deviation around 1% for the faster delivery rate and close to 2% for the slower delivery rate.

Many chromatographic columns have been run in single column runs with a 10 ml syringe set for delivery of 0.110 ml/h, and a high degree of reproducibility of chromatographic patterns with the same substances peaking within the same number of tubes from the start of the column was obtained. This confirms that the reproducibility of the syringe drive, from a practical point of view, is sufficient also at the slow delivery rates.

The syringe drive is specifically designed for low flow rates. The largest syringe available is of 100 ml capacity. One can modify the device and use a bank of 3 syringes; however, this gets unhandy and one would be better off using for higher flow rates a commercially available precision metering pump (Kruger Instruments, San Gabriel, Calif.). This instrument has sufficient accuracy for the higher flow rates, and thus supplements the syringe drive to give coverage up to rates of 600 ml of added eluant per hour.

## TECHNIQUE FOR GRADIENT ELUTION CHROMATOGRAPHY ON A SINGLE COLUMN

The column used is of a special design (Fig. 3). It consists of a piece of glass tubing approximately 18 in. long and with an internal diameter of 6 mm. It is surrounded with a water jacket for thermostatting. At the bottom end a perforated glass plate is melted in to give support for the column and a standard tapered 10/30 joint is fused on at the top. A teflon stopcock with a built-in needle valve (Fischer & Porter Co., Patboro, Pa., No. 80 × 2440 B) is melted to the chromatographic tube below the perforated glass plate.

To pack the column one first connects the water jacket to a constant temperature circulator and the column is then filled three quarters of its length with solvent from the bottom by applying light vacuum to the top. Two circular pieces of filter paper are pushed down with a long glass capillary so that they cover the perforated glass plate. The column is now filled from the top with solvent and an enlarging type of glass adapter with a 10/30 joint at the bottom and a 24/40 joint at the top is put on top of the column. The adapter is filled with solvent and a 250 ml filling funnel with a bottom 24/20 joint is pressed down into the adapter. The tip must be cut off the filling funnel so that it will fit. It is very important at this point that any air bubbles trapped in the adapter be removed. This can be done by introducing a piece of thin teflon tubing through the hole in the stopcock. Trapped air will escape if a few pumping movements are performed.

Approximately 100 ml solvent is now introduced into the filling funnel and the adsorbent is added. It will gradually pass from the filling funnel to the adapter, where it will be whirled around before it slowly settles in the column. The column is tapped gently for about a minute after it has settled and then it is pressed under light pressure. Columns prepared this way are quite uniform in composition and give consistently good separations. The technique is particularly useful in serial analyses where a number of columns can be filled rapidly and conveniently, since the columns can be left to settle after the adsorbent has been added to the filling funnel.

To construct the mixing chamber, a piece of capillary tubing is melted to the bottom of either a Pyrex bottle or an Erlenmeyer flask. A teflon adapter is then turned on the lathe so that it will have at one end a hole that will fit tight around the glass capillary. At the other end it is tapered to fit a hypodermic needle. Another teflon adapter is made to fit the top of the column. It is tapered to fit a 10/30 joint at one end and to fit a hypodermic needle at the other. A collar is left in the middle during the turning of the adapter. This serves as a support for a small stainless steel ring with two hooks. Rubber bands or metal springs secure the adapter to glass hooks on the column.

A piece of teflon spaghetti tubing (Allied Plastics, Elizabeth, N.J.) size AWG 24

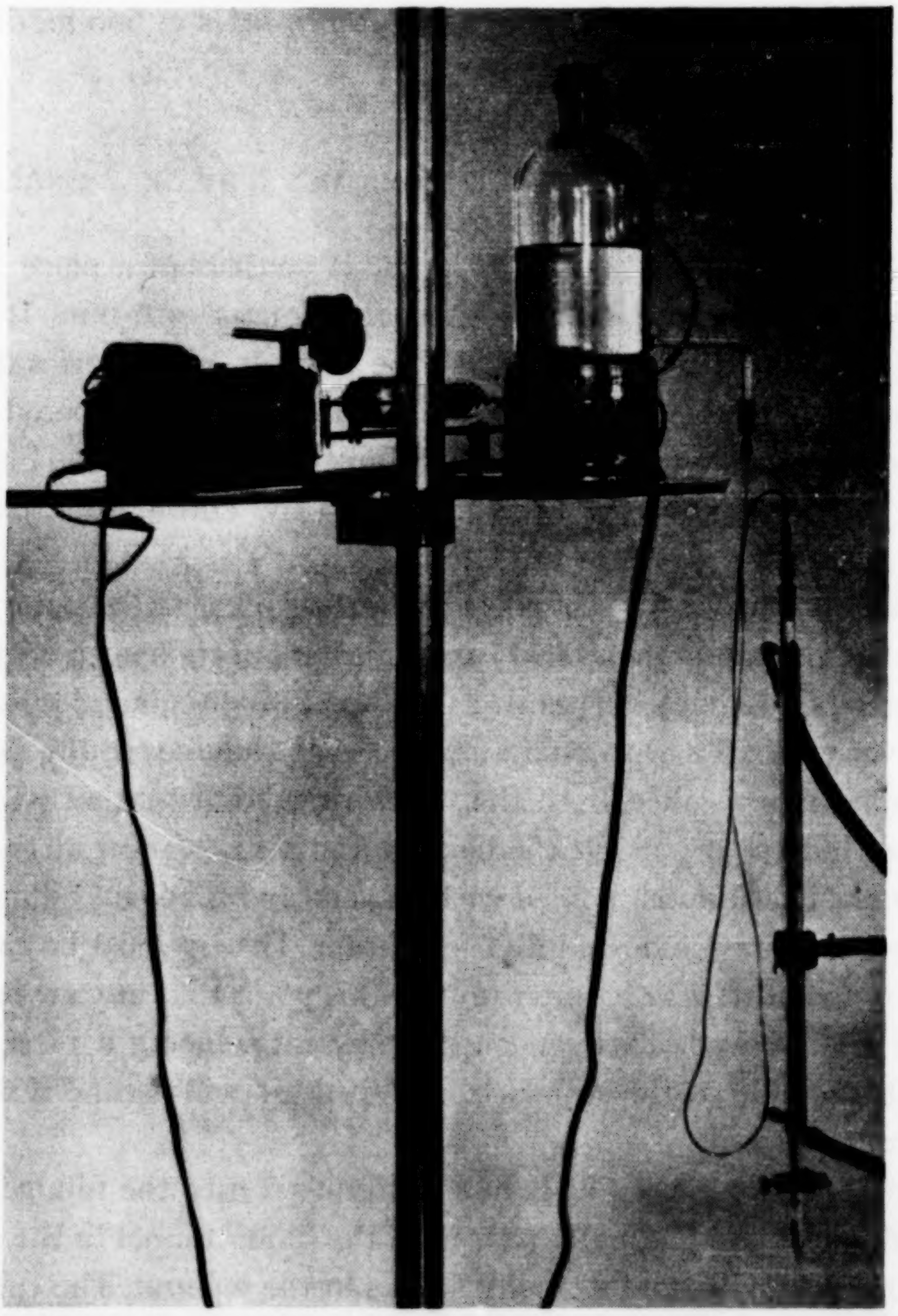

Fig. 3. Technique for gradient elution chromatography on a single column. Mixing chamber, magnetic stirrer and variable speed drive are on a platform sustained by a "polecat". The needle valve stopcock for regulation of flow rate is at the bottom of the column.

of proper length is used to connect two size 20 hypodermic needles. Care must be used to avoid perforation of the teflon tubing with the needle tip when it is attached. The needles are then put on the teflon adapters. The needle size and the diameter of the teflon tubing can be changed if faster flow is needed.

The syringe drive is connected to the mixing chamber through a needle and a piece of teflon tubing. The tubing is carried down into the bottle through a polyethylene stopper in which two small holes have been drilled, one to admit the tubing, the other to serve as a vent.

The mixing chamber is then placed on a magnetic stirrer together with the syringe drive and the whole assembly is placed on a "polecat" platform that can move freely up and down. (Polecat, Inc., Old Saybrook, Conn. manufactures this adjustable aluminum column that is spring-held between ceiling and the floor or tabletop.)

To run a chromatogram on a single column the following steps are taken. The extract to be chromatographed is transferred with a suitable amount of solvent to the column or is transferred to a filterpaper disc which is then put on top of the column. Some solvent is pressed through the column which is then filled. Teflon adapters, needles and teflon tubing are connected together and then connected to the mixing bottle. The teflon adapter fitting the column is lifted to the neck of the bottle and a calculated amount of solvent is added to the bottle. The bottom adapter is now lowered as far as it will reach and tubing and adapter is flushed with a few ml of solvent to remove air from the connecting system. This solvent is added to the mixing bottle to avoid loss. The adapter is then gently pushed down into the 10/30 joint on top of the column. Care must be taken to avoid air bubbles at this stage.

The column is now ready for calibration of flow rate. There is a linear relationship between distance from tip of the column to surface level in the bottle and one can therefore change the flow through the column in a precalculated manner by raising or lowering the platform on the "polecat". It is also possible to set the platform at a height where the outflow from the column is calculated to be somewhat higher than the flow rate needed and then by turning the screw on the needle valve adjust the flow to the proper rate. The first of these methods is the easier and faster one when single columns are run.

The amount of liquid used in the calibration is measured and a similar amount added to the mixing chamber.

The syringe drive is now filled with eluant and the run started by connecting syringe drive and magnetic stirrer to the line and opening the column stopcock.

## TECHNIQUE FOR GRADIENT ELUTION CHROMATOGRAPHY SIMULTANEOUSLY ON SIX COLUMNS

The technique described for single columns is modified so that it can be used for 6 columns (Fig. 4) and is then combined with the technique for simultaneous fraction collection from 6 columns described elsewhere in this issue[3].

One modification necessary to run 6 columns at a time is in the columns. In order that needle valves on the columns are readily accessible for calibration it is necessary to arrange the columns in two parallel rows with approximately 4 in. between each row. Half of the columns are therefore modified slightly by adding a 4-in. S-shaped extension with a ball joint to the tip of the columns. The mixing chamber for the multiple run is a large Erlenmeyer flask with six 10/30 standard tapered female joints melted to the bottom. Teflon adapters similar to those used for the column portions are used to connect to the mixing bottle instead of the type used on the single column mixing bottle.

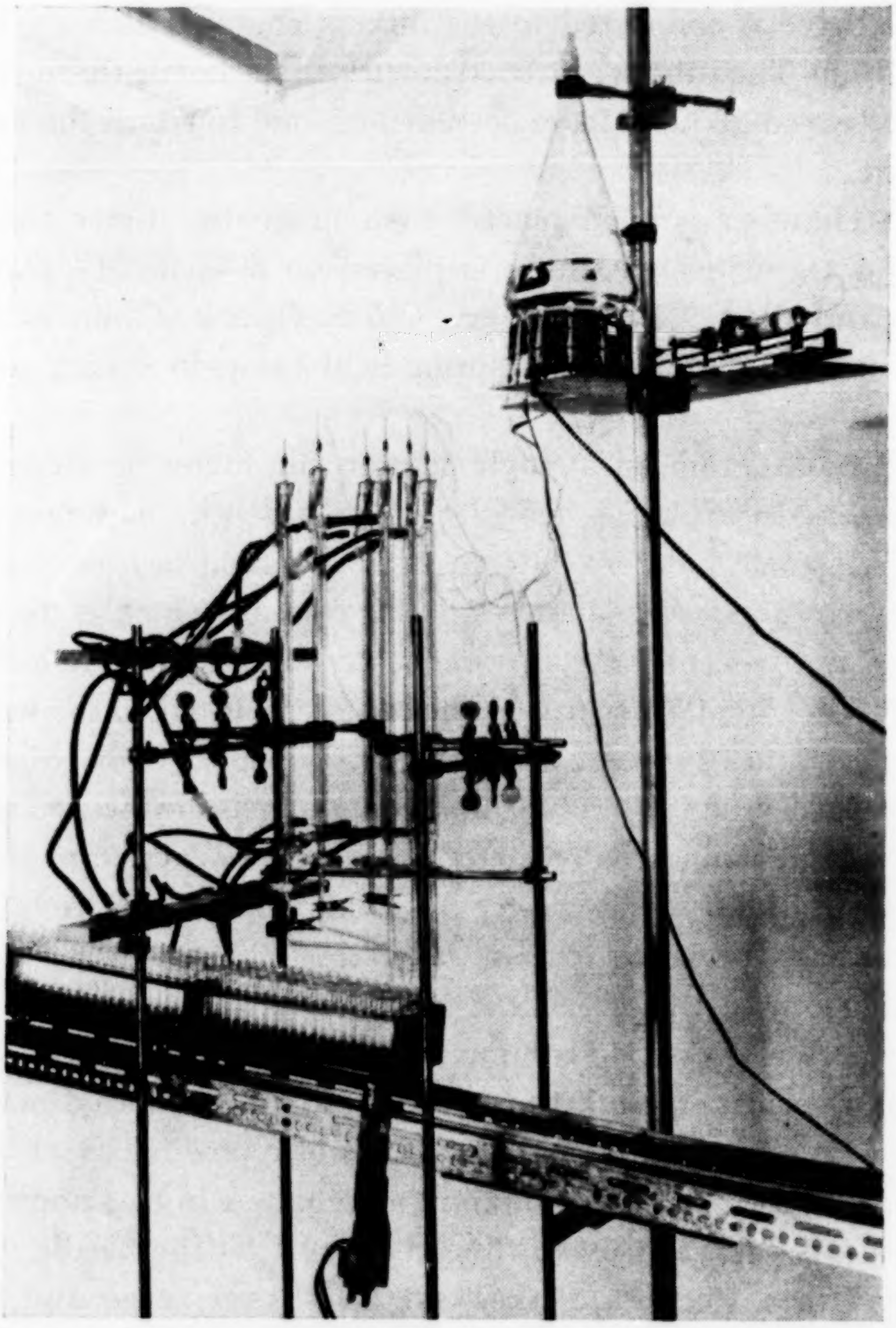

Fig. 4. Technique for gradient elution chromatography on six columns simultaneously. Six columns arranged in two rows and connected to common mixing chamber on "polecat".

The columns are otherwise set up as described under single column runs. All six are connected to the mixing chamber and the columns are ready for calibration of the flow rate.

The optimal conditions for gradient elution chromatography on a single column are first established, as well as the optimal values for the three factors that determine the gradient: outflow from the column, initial volume in the mixing bottle and inflow from the syringe drive have first been experimentally determined. The syringe drive is set to deliver 6 times the flow rate that was found optimal on single column run. The mixing bottle is filled with 6 times the volume of solvent used in single column run, the outflow is then calibrated.

This is accomplished by moving the platform on the "polecat" to a position where the distance between liquid surface in the bottle and the tips of the columns is slightly greater than the one calculated from the calibration charts to give the needed flow through the column. Stopcocks and needle valves are now opened and the flow determined over a 10 min period by collection in measuring cylinders. At the same time measurements are made with a stopwatch to determine the time it takes, for example, for 10 drops to form. The flow per 10 min is read from the measuring cylinders

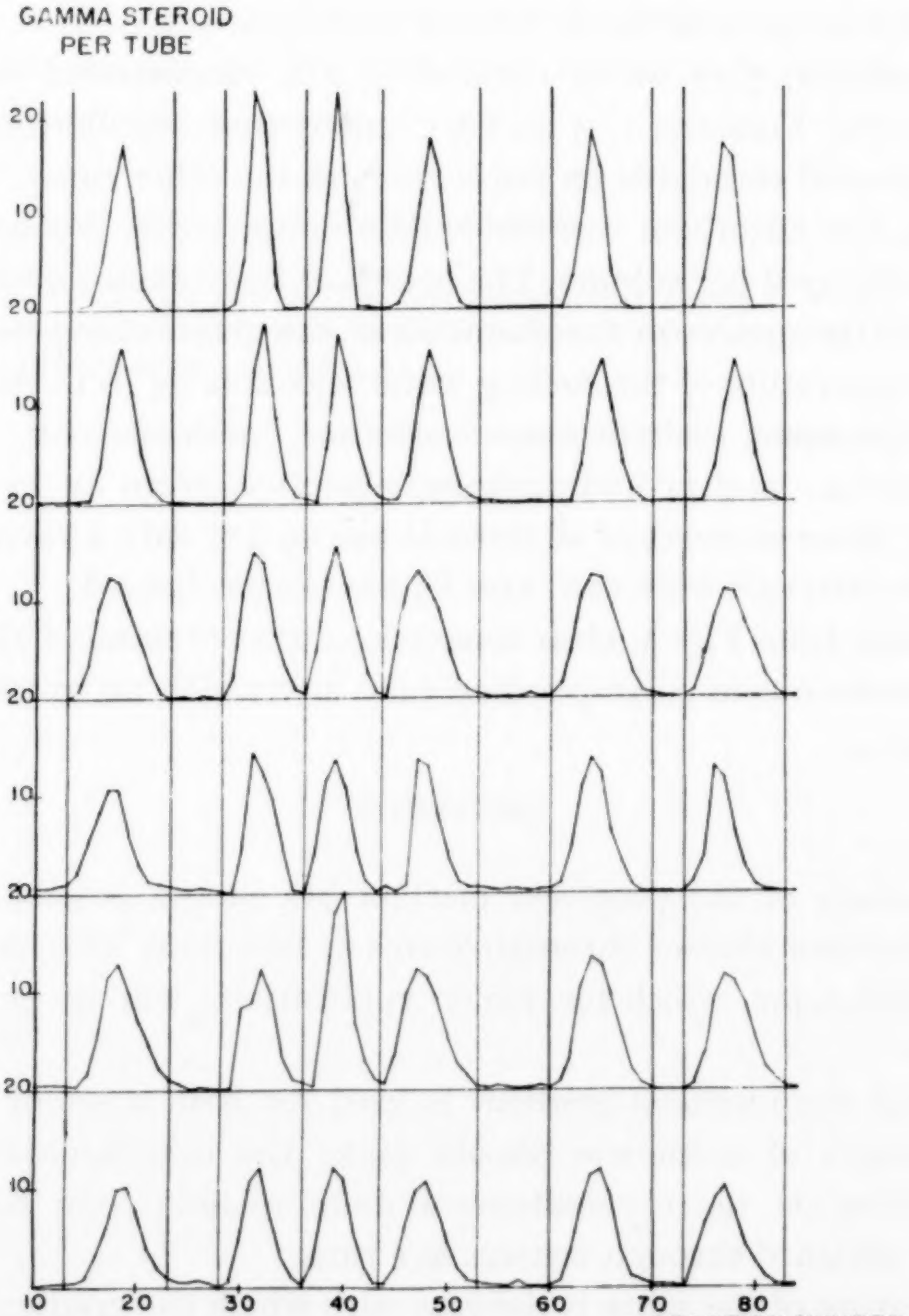

Fig. 5. Simultaneous gradient elution chromatography of 50-$\gamma$ portions of six 17-ketosteroids on six chromatographic columns.

and the change in drop time that should give the needed flow per 10 min is calculated for each column. The initial maximum 10 % difference between the fastest flowing and the slowest flowing column is caused by the non-uniformity of the columns. The differences are now corrected with the needle valves by adjusting the time taken for 10 drops to form to correspond to the calculated flow. The flow from the columns is fairly easily regulated within approximately 2 % difference, not including the possible error on the 10 ml measuring cylinders. From a practical point of view this error was found sufficiently low to give good agreement between runs from the different columns.

## RESULTS

An example of the results obtained when running 6 columns is shown in Fig. 5. A six-column elution chromatogram was run with six 17-ketosteroids present in human urine. The conditions for the run were as follows: starting volume in mixing chamber: 3000 ml pure benzene. Syringe drive set to deliver 0.660 ml/h ethanol from a 50 ml syringe. Distance from column tip to benzene surface at start: 30 in. Flow rate at the start for each column, 5.15 ml/20 minutes ± 2 % (uncorrected for errors in the measuring cylinders). Columns 1, 3 and 5 counted from the bottom were run with transfers of the steroid standards on paper disc; in the other cases, transfers were in benzene solution. The adsorbent was neutral aluminum oxide Woelm with 6 % water added. 11.50 g were used per column. The length of the columns varied from $14^7/_8$ in. to 16 in. owing to differences in the diameter of the glass tubing used for their construction. The temperature of the cooling water was 16.2° ± 0.1°. 50-$\gamma$ portions were run of androstanedione, dehydroisoandrosterone, androsterone, etiocholanolone, 11-ketoetiocholanolone and 11-hydroxyetiocholanolone given in the order of elution from the column. Mean recovery of all steroids was 99.4 % with a variability from 93 % to 106 %. 20 min intervals were used and 85 fractions collected.

As can be seen from Fig. 5, clear separations are obtained of all substances and all the steroids come out in corresponding tube areas with no overlap of areas from different columns.

## DISCUSSION

The main advantage of the proposed method lies in the possibilities it gives for running many gradient elution chromatograms at one time. This should help spread the use of this technique, which for many applications will be superior to simple chromatography.

The fact that the identical gradient is used for both a set of standards and a number of mixtures of unknowns should make the determination of substances from their position on the chromatogram more reliable than determinations on chromatograms obtained through consecutive runs.

A special feature of the setup is the ease with which the gradient can be changed during a run. This means that it is possible to work not only with upwards concave and convex or straight line gradients but also with S-shapes and other shapes. It is possible to bring about such changes in the gradient automatically by coupling a synchronous motor to the screw control and have it monitored from the necessary number of timer-activators.

The proposed technique has been used for a system of analyses for 17-ketosteroids in blood and urine and work is in progress on methods for corticosteroids in blood and urine. Details will be given elsewhere.

## ACKNOWLEDGEMENT

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## SUMMARY

A technique for simultaneous gradient elution chromatography on a number of chromatographic columns is described. The same gradient is used for a set of standards and for mixtures of unknowns. The technique has great flexibility and allows easy change of gradient during a run.

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# EFFECTS OF FIELD STRENGTH AND IONIC STRENGTH ON VELOCITY AND SPREAD OF ZONES IN STARCH BLOCK ZONE ELECTROPHORESIS

GAIL LORENZ MILLER

*Pioneering Research Division, Quartermaster Research and Engineering Center, Natick, Mass. (U.S.A.)*

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As has been implied by TISELIUS AND FLODIN[1] in their review, optimal conditions of field strength and ionic strength in zone electrophoresis represent compromises. This is further brought out in the present study in which the performance of a modified design of starch block zone electrophoresis apparatus was tested. Thus it will be shown that where high fields lead to high velocities, they also lead to undesirable generation of heat; and where the use of low ionic strengths counteracts generation of heat, it also results in disturbed zones. Studies of this nature are especially pertinent to the problem of whether the apparent demonstration by zone electrophoretic procedures of the occurrence of multiple components in enzymes, such as in cellulase[2, 3], might be ascribable to artifacts arising from non-ideal experimental conditions.

## APPARATUS

The principal parts of the zone electrophoresis apparatus used are shown in the photograph in Fig. 1. They consist of a base, A; electrode compartments, B; troughs, C, which contain the starch supporting medium; an interlock switch, D, controlled

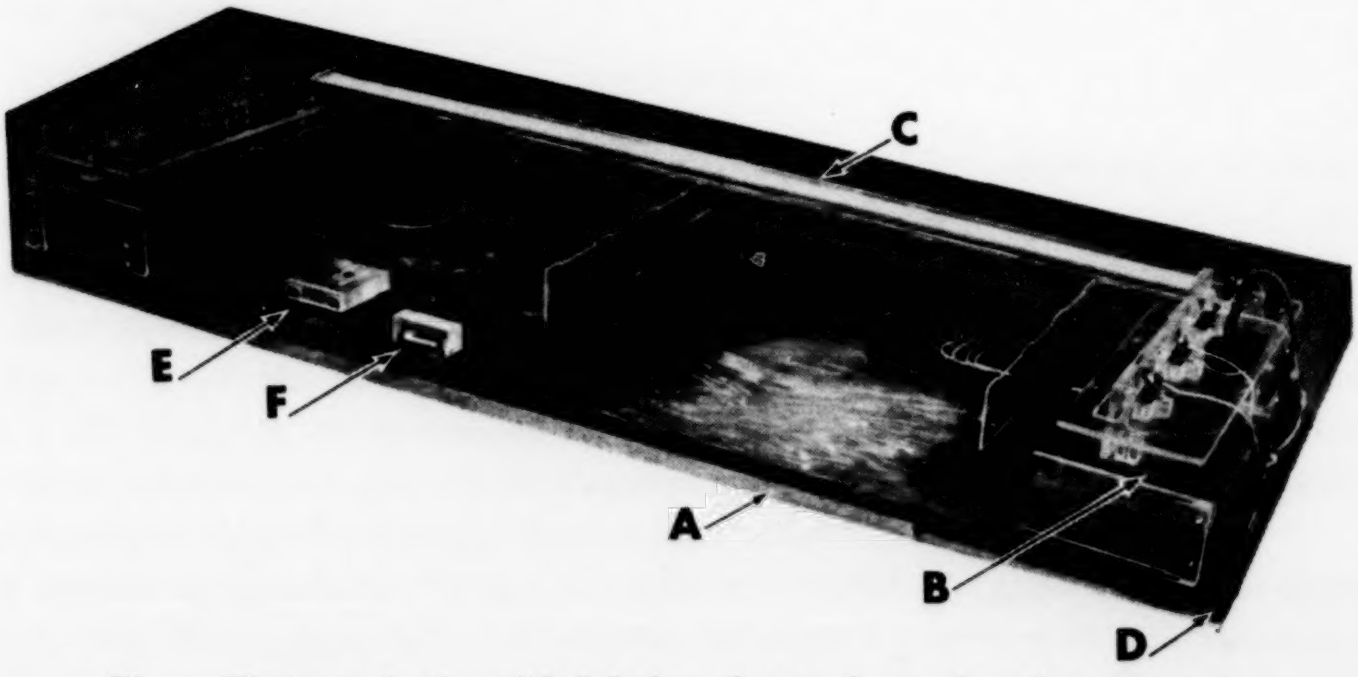

Fig. 1. Photograph of modified design of zone electrophoresis apparatus.

by a safety shield; a cutter, E, for sectioning starch blocks at the ends of runs; and a shaver, F, for trimming the surfaces of the blocks to a uniform level.

The troughs consist of Pyrex plates, 100 × 5.1 × 0.4 cm in dimensions, to which Plexiglas shoulders, 100 × 0.9 × 0.3 cm are cemented with the aid of Goodyear adhesive, Pliobond 30. Plexiglas was used for the shoulders in the belief that edge effects[4] might be reduced by virtue of the low heat conductivity of Plexiglas relative to Pyrex.

A buffer circulating system, a voltage monitoring system, a power supply and electrical distribution system, and a safety shield, also used, are not shown in the photograph. Further description of the apparatus and accessories is given elsewhere[5].

## PROCEDURE

Approximately 110 g of potato starch granules were thoroughly mixed with about 90 ml of buffer. The proportion of buffer to starch was that which would make a slurry which was just loose enough to flow, yet not excessively wet. The ends of the trough were closed off with cellophane adhesive tape and the slurry was then poured into the trough. To remove air bubbles and to pack the starch evenly, the trough was agitated on a vibrating table. The excess buffer which rose to the surface was removed by blotting with folded sheets of filter paper, but care was taken not to remove more buffer than necessary. Excess starch was trimmed to within 3/128 inch of the level of the shoulders of the trough with the aid of the shaver. A wafer consisting of a mixture of 0.1 ml of buffered test sample with an appropriate quantity of starch granules was introduced into a slit prepared in the block midway between the ends. The block was then trimmed to within 1/128 inch of the shoulders, the top glass plate was placed on the block, and good contact was assured with the aid of 6 No. 18 ball joint clamps. The tape was removed from the ends of the block and the block was placed on the rack between the electrode compartments. Cotton cloth wicks were inserted between wick clamp plates and the ends of the block and the block was allowed to equilibrate for about 1 h before the desired voltage was applied. On completion of the run, the block was sectioned, the sections were mixed with definite volumes of water, and the resulting extracts were analyzed for components. Analyses for protein were made by a modification[6] of the method of LOWRY and for polysaccharide by the method of RIMINGTON[7].

## EXPERIMENTAL

Tests of the performance of the modified starch block zone electrophoresis apparatus were first made using 0.1 ionic strength veronal buffer at pH 8.6, a field of 5 V/cm, and test samples consisting of mixtures of 5 mg of bovine plasma albumin, 10 mg of hemoglobin, and 5 mg of dextran. The bovine plasma albumin was obtained from the Armour Laboratories, Chicago, the hemoglobin by hemolysis of washed human red cells, and the dextran from E. T. REESE of these laboratories. All electrophoresis runs were carried out in a cold room at an ambient temperature of 2–4°. Separate runs were made for periods of 2, 4, 8, 16 and 32 h. Visual inspection at the ends of the runs revealed that zones of the hemoglobin were quite even and did not show significant curvature or cometing. Patterns of protein and carbohydrate distribution obtained for the 4-h and 32-h runs are shown in Fig. 2. The progress of the resolution is shown diagrammatically in Fig. 3. The widths of the zones were determined by measuring the distances between baseline intercepts of tangents drawn at inflection points on either side of the peaks. The results shown in the figures indicate relatively linear, disturbance-free migration with time.

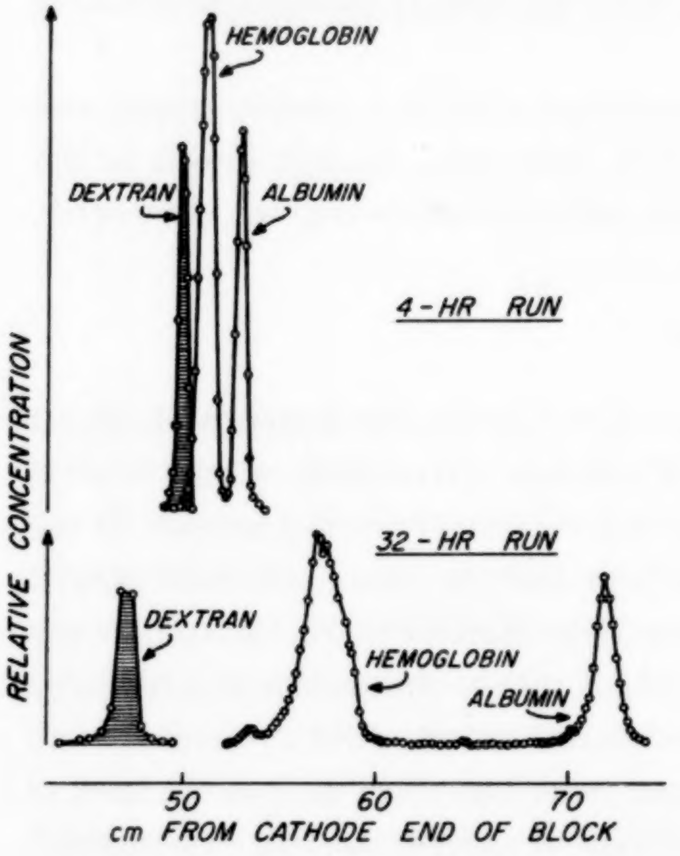

Fig. 2. Electrophoretic diagrams of mixtures of bovine plasma albumin, hemoglobin, and dextran obtained after different periods.

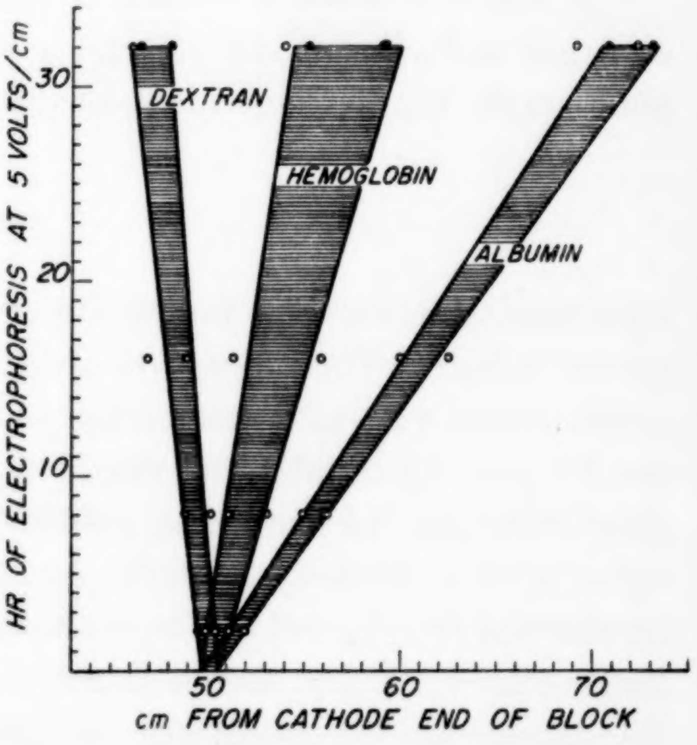

Fig. 3. Progress of resolution during electrophoresis of mixture of plasma albumin, hemoglobin, and dextran. Solid points represent results of duplicate measurements.

Tests were next made to determine whether fields higher than 5 V/cm could be used and whether the expected adverse effects of heat generated at higher fields could be counteracted by the use of low ionic strengths. Fields of 5.0, 7.5 and 13 V/cm and ionic strengths of 0.05, 0.1 and 0.2 were tested, and all runs were made for 80 volt-hours/cm. At intervals during the runs, temperature measurements were made with the aid of thermocouples. Distances of migration, widths of zones, and temperatures of blocks at ends of the runs are summarized in Table I. Distances of migration for the dextran are related to the starting position of the initial zone; distances for the hemoglobin and albumin are related to the final position of the dextran. Also given are distances of migration corrected for viscosity of the buffer and for change of buffer viscosity resulting from change in temperature during the runs. The viscosity corrections are referred to water at 2.5°. Zones which were observed in the runs made at 13 V/cm with buffer of 0.2 ionic strength were too badly disturbed to merit measurement and are not, therefore, evaluated in Table I.

TABLE I

| Field V/cm | Component | 0.05 ionic strength |  |  |  | 0.1 ionic strength |  |  |  | 0.2 ionic strength |  |  |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  |  | Dist. cm | Dist. corr.* cm | Width cm | Temp. °C | Dist. cm | Dist. corr. cm | Width cm | Temp. °C | Dist. cm | Dist. corr. cm | Width cm | Temp. °C |
| 5.0 | Dextran | — 1.37 | — 1.36 | 1.27 | 3.7 | — 1.26 | — 1.27 | 1.23 | 4.3 | — 1.58 | — 1.62 | 1.21 | 5.1 |
|  | Hemoglobin | + 5.82 | + 5.76 | 2.23 |  | + 5.35 | + 5.34 | 1.49 |  | + 4.70 | + 4.83 | 1.45 |  |
|  | Albumin | + 14.91 | + 14.75 | 1.63 |  | + 12.58 | + 12.56 | 1.57 |  | + 9.93 | + 10.19 | 1.40 |  |
| 7.5 | Dextran | — 1.72 | — 1.64 | 1.48 | 4.9 | — 1.32 | — 1.27 | 1.35 | 5.6 | — 0.25 | — 0.24 | 1.35 | 7.1 |
|  | Hemoglobin | + 6.34 | + 6.04 | 3.07 |  | + 5.85 | + 5.61 | 2.09 |  | + 4.60 | + 4.44 | 1.99 |  |
|  | Albumin | + 15.56 | + 14.82 | 1.75 |  | + 13.16 | + 12.61 | 1.43 |  | + 10.03 | + 9.67 | 1.53 |  |
| 13 | Dextran | — 1.98 | — 1.60 | 1.83 | 10.9 | — 2.39 | — 1.61 | 1.52 | 19.4 |  |  |  | 40.4 |
|  | Hemoglobin | + 7.32 | + 5.91 | 3.80 |  | + 7.71 | + 5.22 | 2.60 |  |  |  |  |  |
|  | Albumin | + 17.83 | + 14.40 | 2.14 |  | + 17.87 | + 12.10 | 2.16 |  |  |  |  |  |

* Distances of migration corrected for viscosity of the buffer and for change of buffer viscosity resulting from change in temperature during the runs.

The data show, first of all, that distances of migration of hemoglobin and albumin, per 80 volt-hour/cm, increase with voltage and decrease with ionic strength. The increase with voltage is probably due, however, to the effect of generation of heat on viscosity of the buffer medium since temperature corrections for viscosity eliminate this effect. The decrease with ionic strength which was observed was also noted by TISELIUS AND FLODIN[1]. The distances of migration of the dextran are rather erratic and cannot be correlated well with voltage or ionic strength. This behavior of the dextran may be the result of variable movement of the starting zone during equilibration of the block, which precedes the application of the field.

The widths of the zones of all three test materials increase with voltage and decrease with ionic strength. Visual inspection of hemoglobin zones during runs made with high voltage or low ionic strength revealed marked irregularities in shape, suggesting electrical or mechanical disturbances within the block. Such disturbances presumably extend to some degree to the invisible zones of albumin and dextran. The disturbance with high voltage might be caused by non-uniformity in temperature, giving rise to uneven field, variable viscosity, and convection. Possible causes for disturbances with low ionic strength are not known. It should be pointed out, however, that the effect of ionic strength upon degree of spread is quite dependent upon the nature of the test material, that for hemoglobin being particularly marked, while those for albumin and dextran are very slight. This suggests that disturbances arising from low ionic strength may be more local than general.

Optimal results with the electrophoretic system used, in terms of narrowness and uniformity of zones, are obtained with fields of 7.5 V/cm or lower and with buffer media of 0.1 ionic strength or higher. Under such conditions the spread of albumin with time of migration is only slightly higher than that of dextran while that of hemoglobin is considerably greater. The spread of the hemoglobin peak reflects its known electrochemical inhomogeneity[8].

Distance of migration, *per se*, would not appear to be related to spread to a significant degree since, under favorable conditions of electrophoresis, zones of albumin after migration of 10–13 cm show but slightly greater spread than zones of dextran which have migrated only 0–2 cm. Neither does diffusion appear to be an important factor affecting spread since it was found in separate experiments that glucose and other low molecular weight uncharged materials, having relatively high diffusion rates, show approximately the same spread as does dextran. The possibility is suggested that the spread observed with an uncharged substance might be used as a basis for judging the heterogeneity of charged substances.

## ACKNOWLEDGEMENT

ROBERT BLUM contributed to the design and construction of the apparatus used in this study.

## SUMMARY

Fields that are too high or ionic strengths that are too low may cause disturbances in

starch block zone electrophoresis. With the apparatus used, optimal results were obtained with fields of 7.5 V/cm or lower and with ionic strengths of 0.1 or higher.

Spread of zones does not appear to be related to a significant degree to distance of migration, *per se*, or to diffusion. The possibility is suggested that the spread observed with an uncharged substance might be used as a basis for judging the heterogeneity of charged substances.

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# FURTHER STUDIES ON THE RESOLUTION OF FUNGAL CELLULASE BY ZONE ELECTROPHORESIS

GAIL LORENZ MILLER, ROBERT BLUM AND NOEL F. HAMILTON

*Pioneering Research Division, Quartermaster Research and Engineering Center, Natick, Mass. (U.S.A.)*

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The demonstration by starch block zone electrophoresis of the occurrence of as many as eight electrophoretically distinct, cellulolytically active components in a fungal cellulase preparation[1] raised questions as to whether more refined techniques might reveal additional components or to what degree the observed results might represent an artifact. Studies designed to examine these questions constitute the subject of the present paper. These studies include (1) effect of size of sections into which the starch block is cut, (2) shape and uniformity of zones across the block, (3) reproducibility of enzyme activity measurements, (4) effect of nature of supporting medium, and (5) effect of source of cellulase. To further test the reasonableness of the occurrence of multiple components in cellulase, an attempt was made to construct a hypothetical electrophoretic diagram which was similar to those observed experimentally.

## MATERIALS AND METHODS

Cellulase preparations from three different species of fungi were studied. The preparation most studied originated from a lyophilized stock obtained from a cell-free culture filtrate of *Myrothecium verrucaria* QM 460 which had been grown on cellulose in liquid medium[1]. The other preparations, supplied by F. T. REESE of these laboratories, consisted of an aqueous solution of an acetone precipitate of a filtrate of a shake culture of Basidiomycete QM 806 grown on ground cellulose and an original filtrate of a still culture of *Penicillium pusillum* QM 137g grown on cotton duck strips.

The zone electrophoresis was carried out with the aid of starch blocks 100 × 3.3 × 0.3 cm in size according to procedures described elsewhere[2,3]. The conditions of ionic strength and field strength which were used were based on the results of studies with known test materials[3]. The buffer was 0.1 ionic strength sodium phosphate at pH 7, the field 8 V/cm, and the duration of the runs 40 h.

The test samples consisted of 0.1 ml aliquots of solution of cellulase containing around 100 units of the enzyme. Enzyme activities of the test samples were determined by a modification of the method of REESE, SIU AND LEVINSON[4]. For convenience, however, data for the distribution of enzyme activity in the zone electrophoretic experiments were recorded directly as arbitrary units of absorbance of the colored solutions produced in the tests, without conversion to units of activity. Dextran was used as a marker in certain of the tests. It was measured by the method of RIMINGTON[6].

## EXPERIMENTAL

### *Size of sections into which block is cut*

When starch blocks at the ends of runs were cut into 1-cm pieces and the pieces were extracted with water and analyzed for enzyme activity, the results were very similar to those reported previously, using a starch block of different dimensions[1]. Nine fairly distinct components could be seen, although no one of them was completely separated from all of the others. When the blocks were cut into 0.5-cm sections, however, five further components were revealed to give a total of fourteen. This is shown by the diagrams in Fig. 1 for two different tests. The dotted lines are drawn to show correspondence between components. The shaded peaks represent dextran used as a marker. When the blocks were cut into 0.25-cm sections, further components were revealed to give a new total of at least twenty-four, shown by the diagrams in Fig. 2.

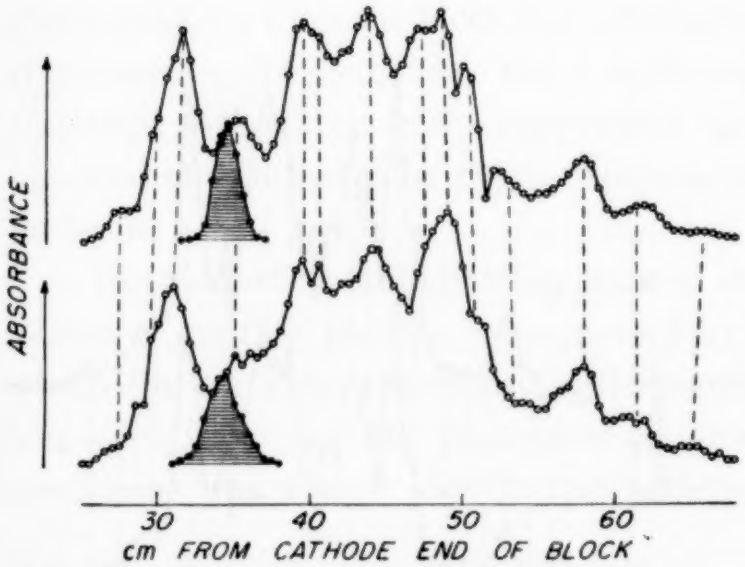

Fig. 1. Electrophoretic diagrams of *Myrothecium verrucaria* cellulase for blocks cut into 0.5-cm sections. Upper diagram, test 1; lower diagram, test 2.

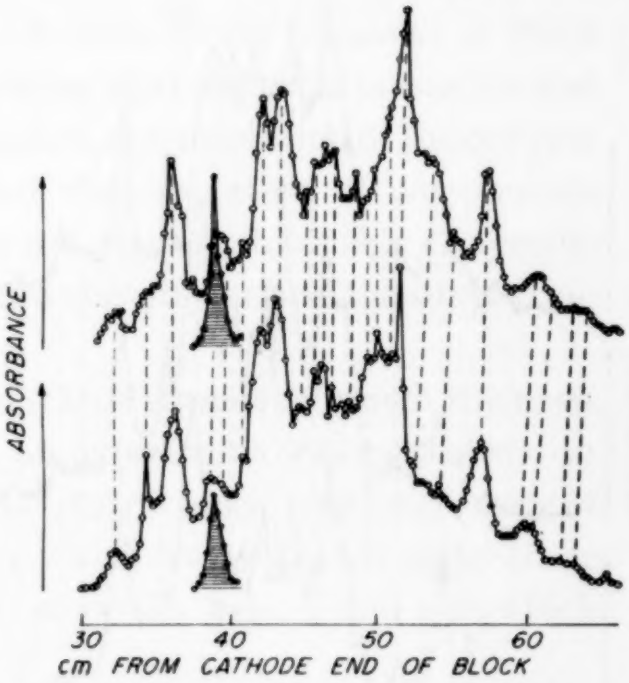

Fig. 2. Electrophoretic diagrams of *Myrothecium verrucaria* cellulase for blocks cut into 0.25-cm sections. Upper diagram, test 1; lower diagram, test 2.

The significance of the multiplicity of components revealed in the foregoing tests was supported by the reproducibility of the results, a total of fourteen tests having been made with blocks cut into 1-cm sections, three tests with blocks cut into 0.5-cm sections, and four tests with blocks cut into 0.25-cm sections. It was also supported by the results of a large number of additional tests not reported in this paper in which ionic strength, voltage, and mode of application of sample were varied[6]. The degree to which heights and positions of peaks varied in the different runs was probably due to slight variations in techniques for preparing the starch blocks and for analyzing the fractions which were instituted from time to time as the work progressed.

### *Shape and uniformity of zones across the block*

To determine whether curvature or cometing of zones occurred under the conditions of electrophoresis used and whether such behavior might to some degree be responsible for the appearance of multiple components in cellulase, experiments were carried out in which the blocks at the ends of the runs were, in effect, cut into six 100-cm long strips as well as into 0.5-cm transverse sections. The diagrams which were obtained for the first three of the six strips are shown in Fig. 3. Those obtained for the remaining three strips, not shown, were found to be very similar. It can be seen that matching of peaks was generally very good, indicating that the curvature of the zones was too slight to be a significant cause of artifacts.

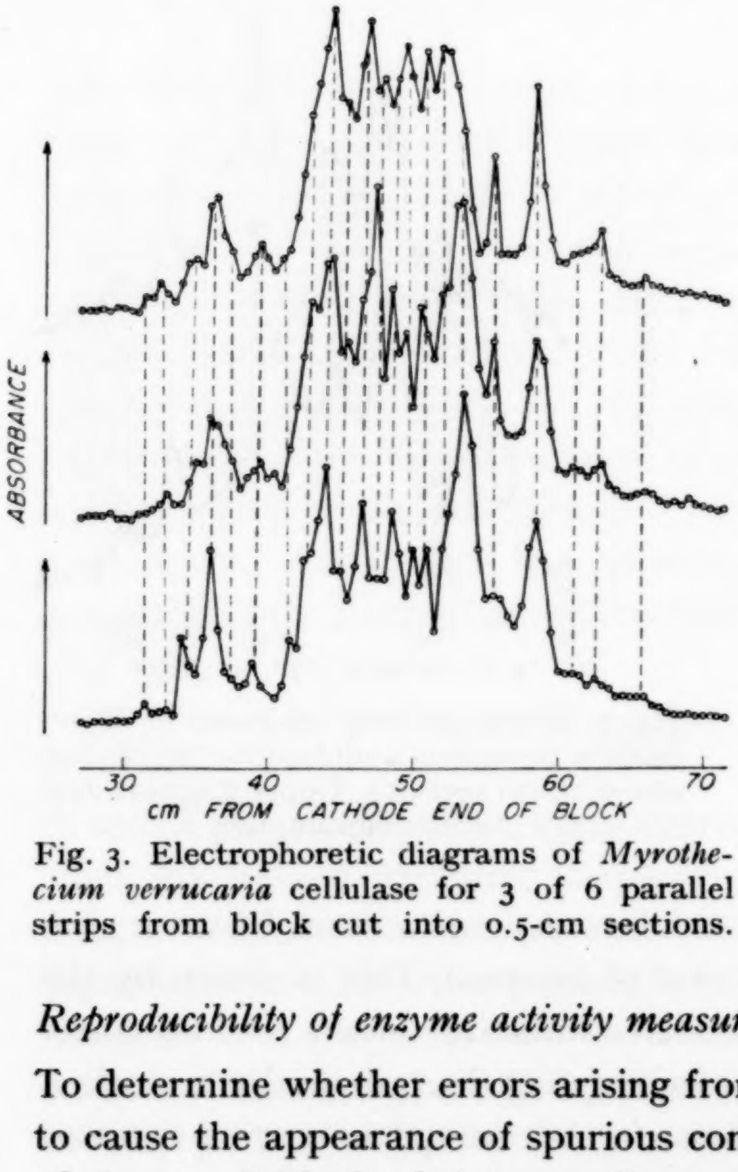

Fig. 3. Electrophoretic diagrams of *Myrothecium verrucaria* cellulase for 3 of 6 parallel strips from block cut into 0.5-cm sections.

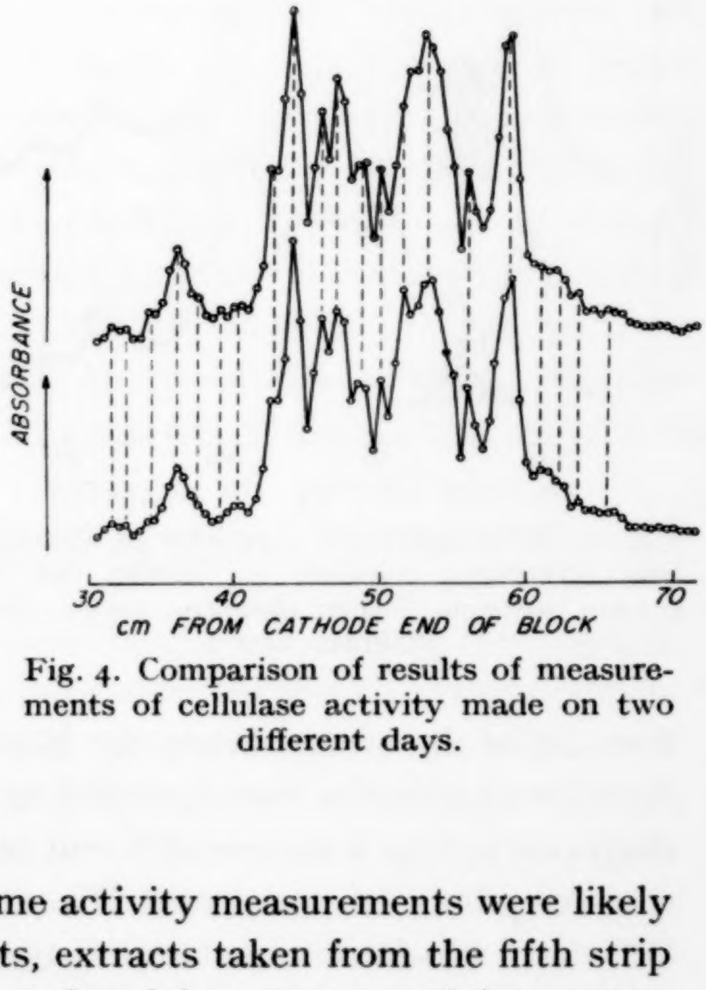

Fig. 4. Comparison of results of measurements of cellulase activity made on two different days.

### *Reproducibility of enzyme activity measurements*

To determine whether errors arising from enzyme activity measurements were likely to cause the appearance of spurious components, extracts taken from the fifth strip of the starch block of the preceding test were analyzed for enzyme activity on two different days. The results, shown in Fig. 4, demonstrate that matching of peaks was highly adequate in every instance and that, although exact reproducibility of individual data was not attained, in few cases did peaks appear or disappear due to errors in activity measurement.

### *Nature of supporting medium*

Earlier work indicated that cellulase was not adsorbed to any detectable degree by the starch used as a supporting medium[1]. Because of the possibility, however, that even a slight affinity of cellulase for starch might give rise to peculiar chromatographic effects manifesting themselves in the form of multiple components, zone electrophoresis experiments were carried out with other types of supporting media. It was not to be expected that different supporting media would show similar chromatographic effects, assuming that such effects could exist. Blocks made up with glass beads[7], powdered polyvinyl chloride Geon-426[8], and powdered nylon were tried. Results with the glass beads were unsatisfactory because of excessively high electro-endosmotic rates. With the polyvinyl chloride the endosmosis was also quite high, but this could be reduced by placing several layers of cellophane dialysis membrane over the ends of the block. Patterns of enzyme activity obtained when either polyvinyl chloride or powdered nylon were used as supporting media were strongly reminiscent of those obtained when the starch granules were used, but the different cellulolytic components were observed to be much less well resolved. The dextran peaks obtained in these tests also were found to be abnormally diffuse, indicating that electrical or mechanical disturbances within the block had partially counteracted the resolution of the enzyme components. Nevertheless, the results suggest that the resolution of components observed with starch as the supporting medium are not attributable solely to specific chromatographic or other types of interaction between the enzyme and the supporting medium.

In an effort to exclude adsorption of enzyme on starch granules through the mass action of another protein, as suggested by MICHL[9], an experiment was carried out in which the 0.1 ionic phosphate buffer medium in the starch block contained 2 mg of hemoglobin per ml. The pattern of enzyme activity which was obtained under these conditions was almost identical with those already shown in Figs. 1 and 2 in which

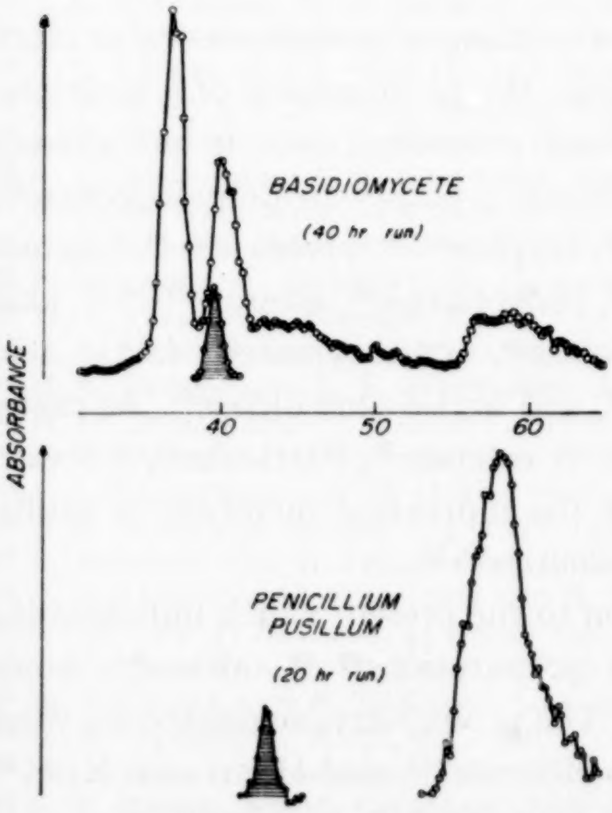

Fig. 5. Electrophoretic diagrams of cellulases from Basidiomycete and *Penicillium pusillum*.

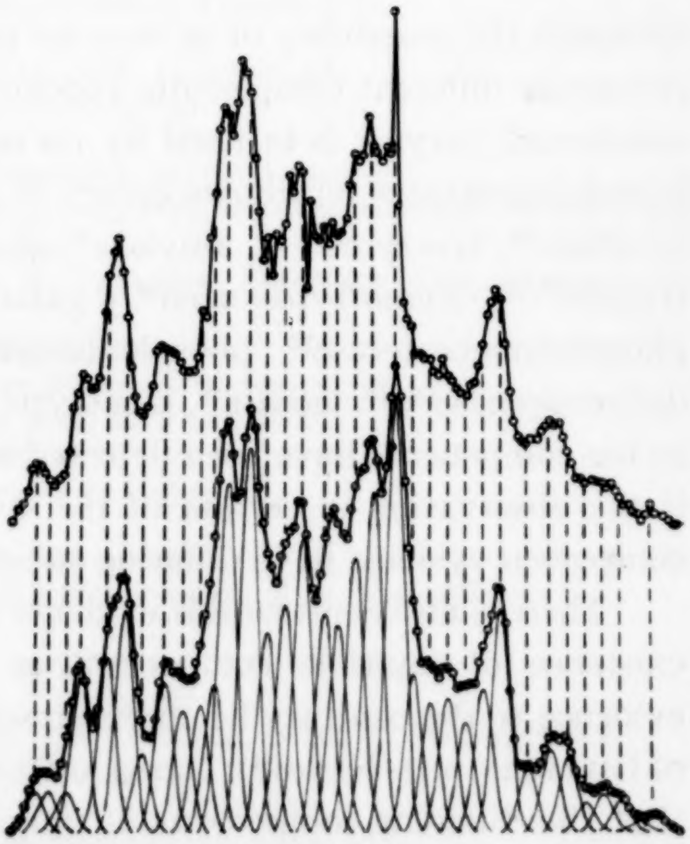

Fig. 6. Comparison of hypothetical and experimental electrophoretic diagrams. Upper diagram, experimental; lower diagram, hypothetical.

hemoglobin was not present, thus providing additional evidence that the appearance of multiple components was not the consequence of chromatographic effects.

### *Cellulase preparations from different fungi*

Patterns obtained for cellulases from two organisms other than *Myrothecium verrucaria* are shown in Fig. 5. It appears possible, although not certain, that some of the components of the cellulases from *Myrothecium verrucaria* and the Basidiomycete are qualitatively identical, based on similarities in distances of migration. On the other hand, the components present in the cellulase from *Penicillium pusillum* appear to migrate altogether faster than those of the other fungi. Since the presence of artifacts might be expected to result in a similarity of patterns of cellulases from different sources, the observed difference in patterns can be taken as evidence for the absence of artifacts.

### *Construction of hypothetical electrophoretic diagram*

It might be questioned whether under the conditions used it is possible to obtain the observed degree of resolution for such a large number of components. This question was tested by constructing an electrophoretic diagram consisting of a hypothetical mixture of 35 components, migrating at rates such that the centers of their zones would be 0.75 to 1.25 cm apart, having band widths comparable to that shown by dextran[3], and possessing selected enzyme activities. The result obtained is shown in the lower pattern of Fig. 6. The similarity of the hypothetical pattern to an experimental pattern shown above it indicates that the observed resolution is reasonable.

## DISCUSSION

Although the possibility of an enzyme preparation containing as many as 24 or more physically different components appears exceptional, the phenomenon of a multiple-component enzyme is in itself by no means without precedent since it has already been demonstrated for ribonuclease[10–16], glyceraldehyde-3-phosphate dehydrogenase[17], invertase[18], lysozyme[19, 20], amylase[21], peroxidase[22], trypsin[23, 24], protease[25, 26], chymotrypsin[27, 28], chymotrypsinogen[28], β-galactosidase[29], phosphatase[30], esterase[21, 26, 31], acid phosphomonoesterase[32], phosphodiesterase[33], enolase[34], maltozymase[35], lactic acid dehydrogenase[36, 37], laccase[38], hemolytic enzyme[39], and aryl-β-glucosidase[40]. As many as ten components have been detected in mouse liver esterase[31]. Particularly relevant is the observation in certain of the studies that the individual members of multicomponent systems have differing substrate specificities[30, 35].

There is also considerable evidence in addition to the present which indicates the existence of multiple components in cellulase preparations[40–44], although some evidence to the contrary has been presented [45–47].The probability, suggested by work of GILLIGAN AND REESE[41], GRIMES, DUNCAN AND HOPPERT[42], and HASH AND KING[40], that the different components of fungal cellulase possess differing specificities with respect to cellulose in its different forms or stages of degradation merits further investigation. The isolation and study of pure individual components should provide definitive answers to the question.

## SUMMARY

Refinement in technique has served to show the presence of twenty-four or more electrophoretically distinct, cellulolytically active components in a fungal cellulase preparation. Studies of uniformity of widths of zones, reproducibility of activity measurements, nature of supporting medium, source of cellulase, and construction of hypothetical electrophoretic diagrams support the findings.

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# Short Communications

## Response of the $\beta$-ray ionization detector to unesterified lower fatty acids in gas-liquid chromatography

According to the theory of the $\beta$-ray detector given by LOVELOCK[1], response per unit mass for any molecular species will decrease strongly with rising molecular weight up to a molecular weight of about 100, and tend to a constant level at values over 200. This theory was moderately well borne out by the compounds tested by LOVELOCK.

Before using the $\beta$-ray detection method for a quantitative analysis of mixtures of monobasic saturated straight-chain fatty acids (4–10 carbon atoms) in the unesterified state it was necessary to check LOVELOCK's theory for these acids, since his experiments included fatty acid methyl esters but not free fatty acids.

Our stationary phase was a polyester of maleic acid, adipic acid and ethylene glycol (see BÖTTCHER *et al.*[2] for details), which gave well-defined peaks, with much less tailing than was obtained on a mixture of Apiezon L (80 %) and behenic acid (20 %). The apparatus and $\beta$-ray detector were supplied by Messrs. W. G. Pye and Co., Ltd., Cambridge, England. The potential across the detector was 1000 V and the carrier gas argon, at a flow rate of 35 ml/min, the outlet of the column being at atmospheric pressure. Column temperature was 175°, and sample sizes were of the order of 0.02 $\mu$l.

The values for the peak areas relative to that for pelargonic acid per unit mass and per mole, obtained from our measurements on known mixtures of pure acids, are shown in Table I.

TABLE I

PEAK AREAS OF FATTY ACIDS PER MOLE AND PER UNIT MASS
(Relative to those of pelargonic acid)

| *Compound* | *Relative peak area per mole* | *Relative peak area per unit mass* |
|---|---|---|
| Butyric acid ($C_4$) | 0.09 | 0.16 |
| Valeric acid ($C_5$) | 0.35 | 0.54 |
| Caproic acid ($C_6$) | 0.53 | 0.72 |
| Enanthic acid ($C_7$) | 0.74 | 0.90 |
| Caprylic acid ($C_8$) | 0.86 | 0.94 |
| Pelargonic acid ($C_9$) | 1.00 | 1.00 |
| Capric acid ($C_{10}$) | 1.09 | 1.00 |

The relative peak areas per unit mass are plotted against molecular weight in Fig. 1. In the same figure the dotted line indicates the values according to LOVELOCK's formula[1]. Instead of a decrease of the areas per unit mass with increasing molecular weight, as predicted by the latter formula, we find with the fatty acids a marked increase for molecular weights up to 150. The curve shows a tendency to reach a constant value for the higher molecular weights.

Similar anomalous behaviour with this type of detector has been observed by LOVELOCK *et al.*[3] for highly halogenated and nitro-compounds, and this was attributed to the capture of free electrons by the relevant groups. The same effect may be operative in the case of the carboxyl group of the free fatty acids. We have evidence that this phenomenon may be observed for other compounds with a large proportion of carbonyl oxygen in the molecule: for example, the peak area per mole of dimethyl succinate ($C_4$) is only about 0.4 times that of dimethyl sebacate ($C_{10}$).

The effect is not attributable in any way to trans-esterification of the acids on the polyester stationary phase, since similar results were obtained on the Apiezon–behenic acid phase mentioned above, although they could not be made quantitative because of excessive tailing.

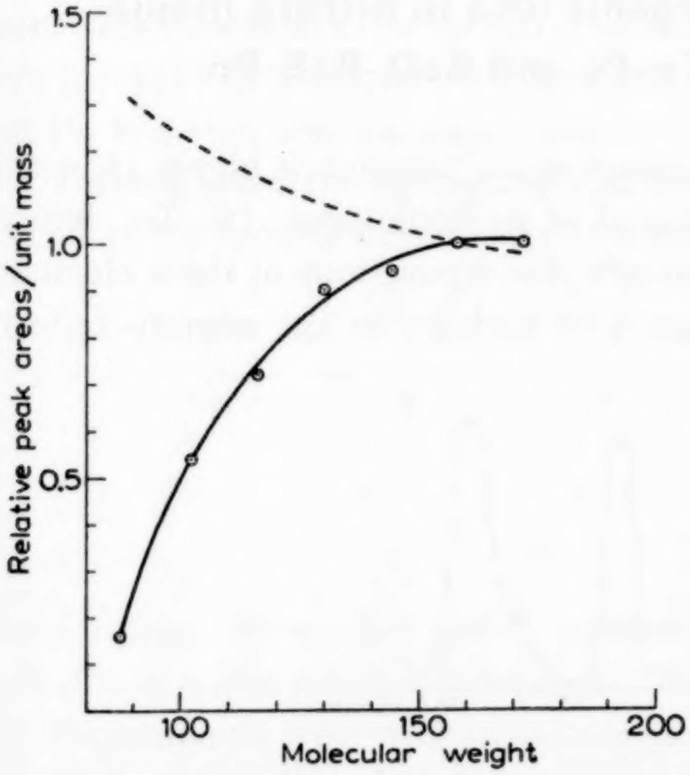

Fig. 1. Observed peak areas per unit mass of monobasic straight-chain saturated fatty acids (relative to that of pelargonic acid) as a function of molecular weight. The dotted curve indicates the relationship predicted by LOVELOCK's formula[1].

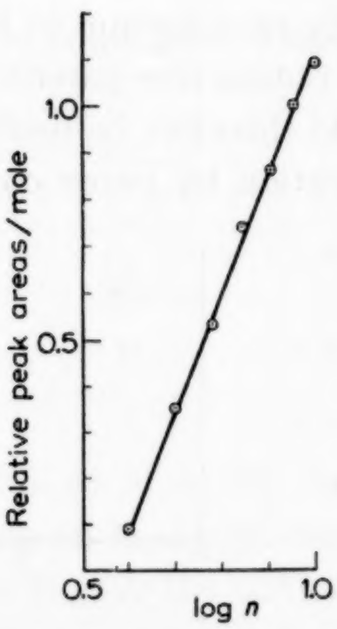

Fig. 2. Observed peak areas per mole of monobasic straight-chain saturated fatty acids (relative to that of pelargonic acid) as a function of $\log_{10}n$, where $n$ is the number of carbon atoms in the molecule.

The relative peak areas $A_r$ per mole of the investigated fatty acids follow quite closely the empirical equation:

$$A_r = 2.5 \log_{10}n - 1.41$$

where $n$ is the number of carbon atoms in the molecule, and the peak area per mole of pelargonic acid is again chosen as unity. This relationship is shown in Fig. 2.

These results indicate that careful calibration of the apparatus is necessary for compounds of molecular weight less than 100; on the other hand such effects may be useful in indicating the nature of unknown compounds in mixtures.

*Department of Physical Chemistry, University of Leiden*
*(The Netherlands)*
C. J. F. Böttcher
G. F. G. Clemens
C. M. van Gent

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# Paper chromatography of inorganic ions in nitrate media
## II. Separation of Se–Te–Po and RaD–RaE–Po

The tendency of polonium to form nitrate complexes in solution is higher than that of its usual radioactive parents (RaD–RaE) and of its homologues (Se–Te). Nitrate media should therefore be useful for chromatographic separations of these elements.

A separation by paper chromatography of Se and Te, in the selenite–tellurite

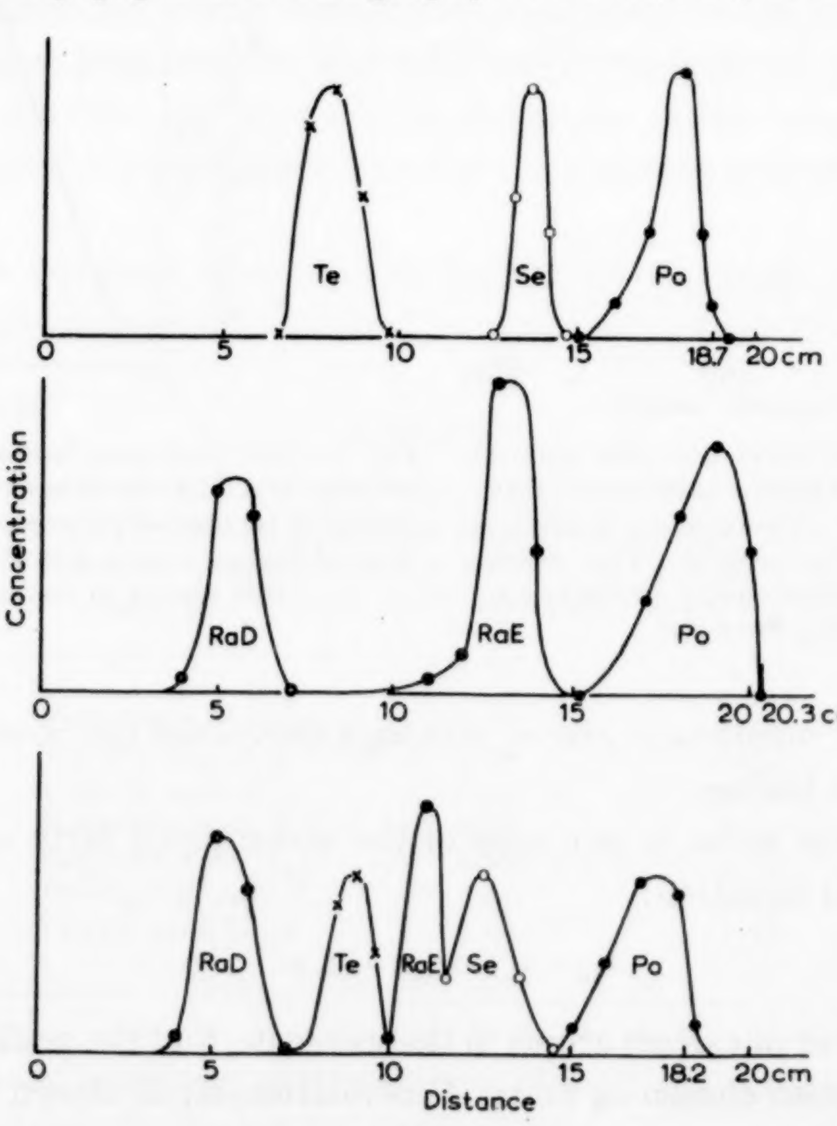

Fig. 1.

form, has been obtained in nitric acid media with butanol–methanol[1]. The $R_F$ values for Pb and Bi in nitric acid with various solvents[2] differ sufficiently to allow quantitative separations of these elements.

In a first series of experiments using several alcohols as solvents, we observed that the separations in nitric acid media are not very efficient since the $R_F$ value for polonium is close to that obtained for selenium.

As was observed with the rare earths[3], the addition of concentrated lithium nitrate to the nitric acid solution increases the $R_F$ of polonium, and thus makes efficient separations possible.

The chromatograms given in Fig. 1 were obtained on Whatman paper No. 1 with a development time of 18 hours at room temperature. A mixture of butanol (50%) and propanol (50%) previously shaken with a solution of $7\,M$ $LiNO_3$ + $2\,M$ $HNO_3$, was used as solvent.

Se and Te as selenite and tellurite were detected by spraying with $SnCl_2$ and the spots were measured in an optical densitometer. RaD was detected through its gamma rays (47 keV) in a scintillation spectrometer. RaE was measured in a G.M. counter and Po in a thin window mica counter.

Table I gives the $R_F$ values obtained under these conditions. These values are refered to the second front of the solvent.

TABLE I

| *Element* | *RaD* | *Te* | *RaE* | *Se* | *Po* |
|---|---|---|---|---|---|
| $R_F$ | 0.27 | 0.47 | 0.62 | 0.72 | 0.97 |

These results show that good separations are obtained of Se–Te–Po and RaD–RaE–Po. It is also possible to separate all these five elements in a single chromatogram.

Experiments with weighable quantities of Pb and Bi gave the same $R_F$ values as obtained with RaD and RaE.

*Brazilian Centre for Physics Research**,
*Rio de Janeiro (Brazil)*

M. C. Levi
J. Danon

[1] F. H. Burstall, G. R. Davies, R. P. Linstead and R. A. Wells, *J. Chem. Soc.*, (1950) 64.
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* Centro Brasileiro de Pesquisas Físicas, Avenida Wenceslau Bras 71, Rio de Janeiro.

# A new type of tank for paper microelectrophoresis of proteins from biological fluids

The electrode vessels in the apparatus described here serve at the same time for stretching the paper strips. The construction of the entire unit is so simple that it can be easily assembled in any laboratory.

The tank serving as wet cell for electrophoresis consists of two vinidur photographic cuvettes 525 mm by 625 mm. A shallow dish with water placed on the bottom of the tank provides moisture.

A vinidur photographic cuvette was used for the first time for this purpose by MONCKE (according to BÜCHNER AND GABSCH[1]) but he covered the cuvette with a glass plate. We use for the same purpose a second photographic cuvette of the same size but inverted, the bottom of which is replaced by a glass plate in a metal frame inclined at about 20° as a protection against drops of condensed water falling on the

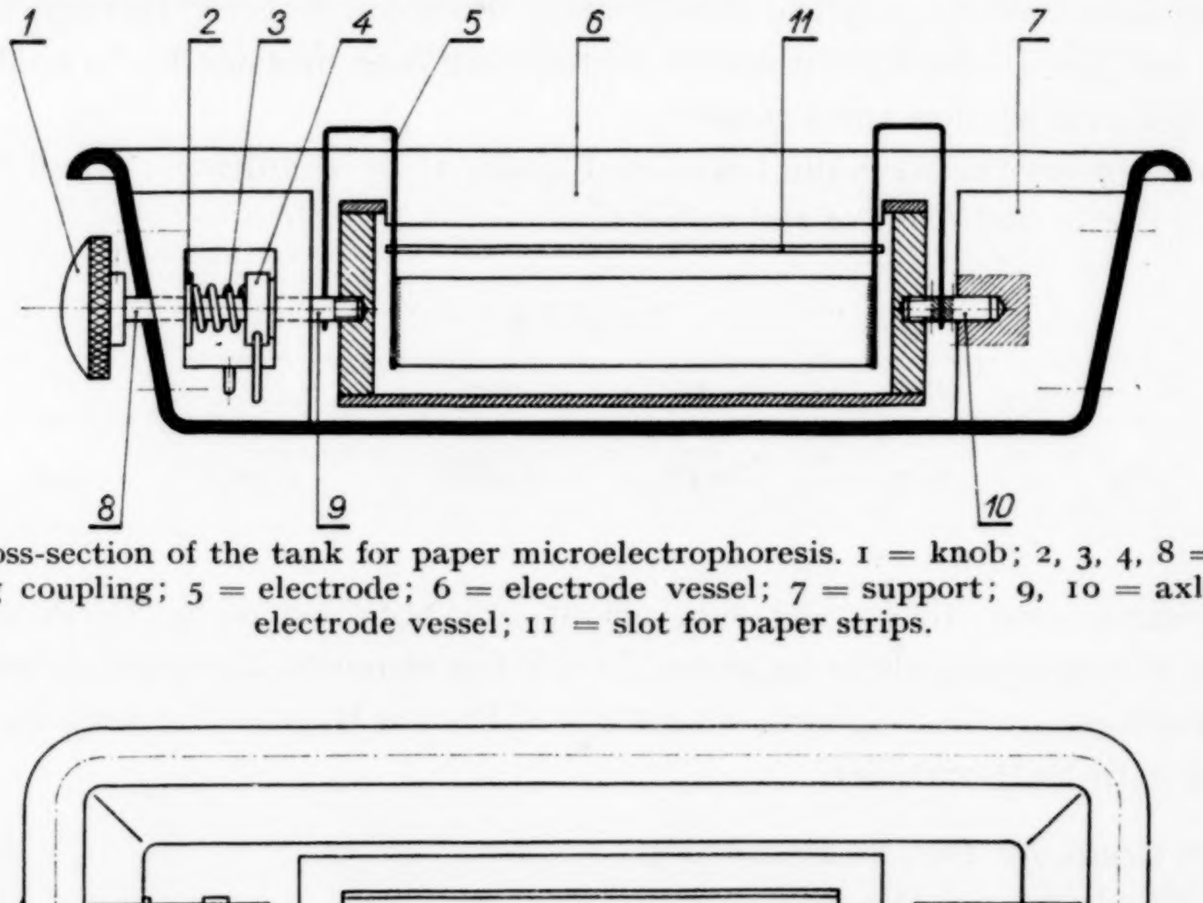

Fig. 1. Cross-section of the tank for paper microelectrophoresis. 1 = knob; 2, 3, 4, 8 = parts of the spring coupling; 5 = electrode; 6 = electrode vessel; 7 = support; 9, 10 = axles of the electrode vessel; 11 = slot for paper strips.

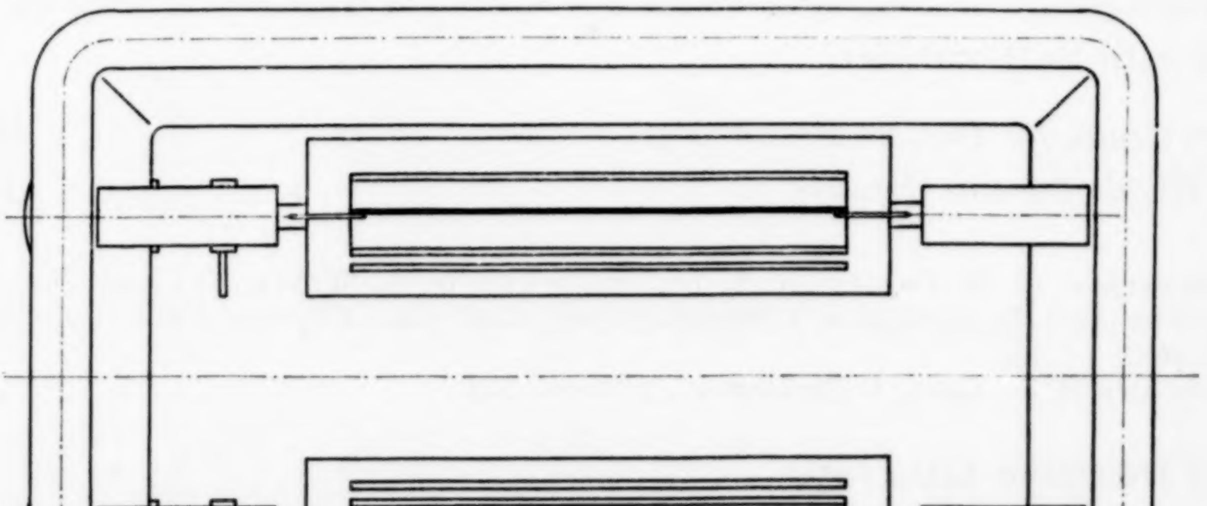

Fig. 2. Horizontal projection of the tank for paper microelectrophoresis.

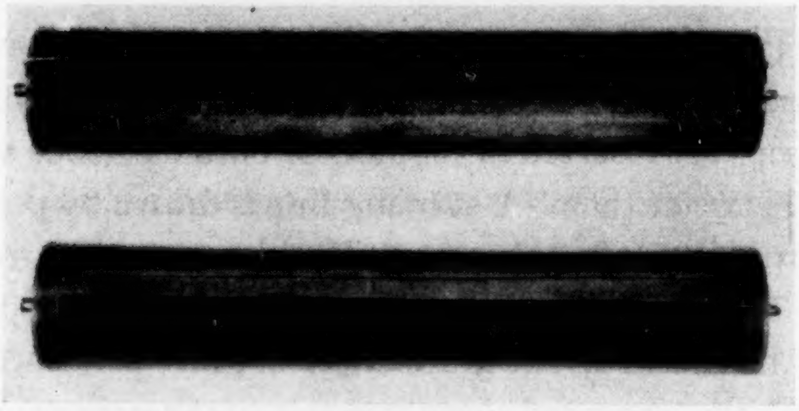

Fig. 3. Electrode vessel (the upper vessel with electrode).

strips. The electrode vessels are suspended on special vinidur supports on the sides of the tank. The vessels are provided with knobs for turning and with spring couplings which allow fixation of the vessels and their easy removal (Figs. 1 and 2).

*Electrode vessels*

These are designed to hold and at the same time to stretch the paper strips (Figs. 3 and 4). Thus special frames for holding the strips, which so often tear the wet paper, are unnecessary. The vessels are made of vinidur tubing 50 mm in diameter and 380 mm long. Both ends of the tube are closed with a vinidur disc 50 mm in diameter and 10 mm thick. The discs are sealed on the tube with vinidur glue Pc 2, which gives absolute security against leakage.

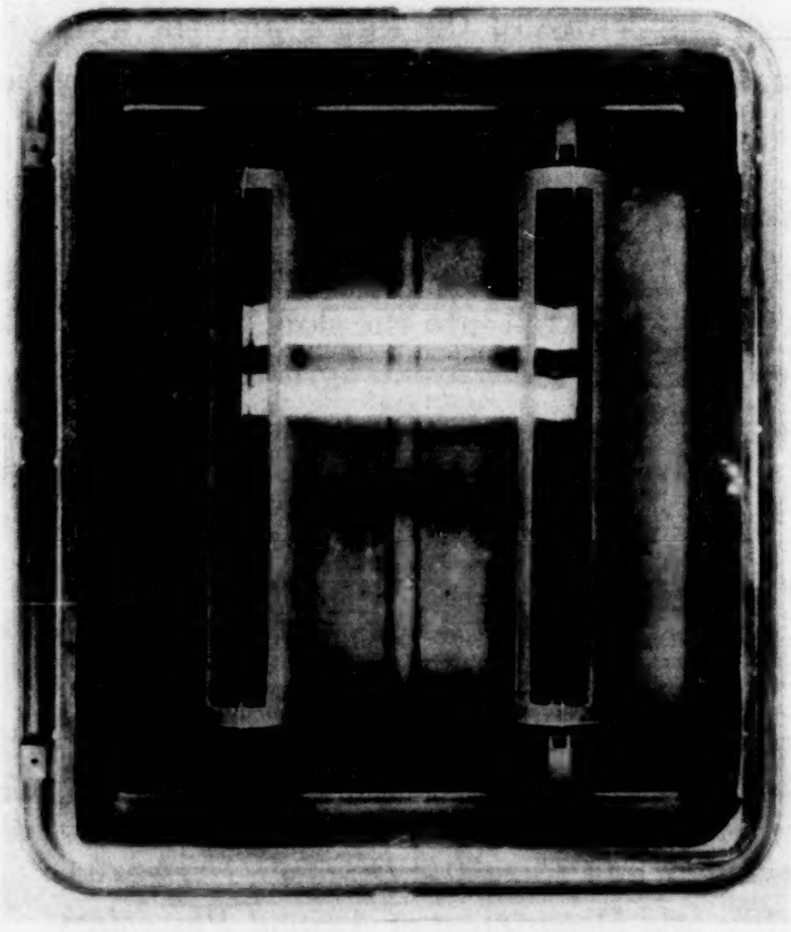

Fig. 4. Microelectrophoresis tank with stretched paper strips (seen from above).

The tubes are provided with an opening in their upper side. This opening, 30 mm by 370 mm, serves to fill, empty and clean the vessel and to put in the electrode. Each vessel has a volume of approx. 300 ml.

A narrow slot, 370 mm long and 1 mm wide, is cut out in the side of the tube at a distance of about 7 mm from the longer edge of the opening and parallel to it. The ends of the paper strips are inserted in the slots of both electrode vessels and the strips are then stretched between them. A starting line is drawn by pencil on the strips at a distance of 8.5 cm from the end of the strip[2]. The slots can hold 10 strips of 3 cm width and 28 cm length. With a medinal–acetic acid[2] buffer and a potential gradient of 5 V per cm of length and a current gradient of 0.3 mA per cm of width the protein fractions of the serum are separated along a section of the strip measuring approx. 9 cm.

### *The electrodes*

The electrodes are made of highly resistant stainless steel produced in Poland*. They measure 40 mm by 360 mm with a rather large surface of 144 cm$^2$. After polishing they are apt to become corroded by concentrated hydrochloric acid, therefore no buffer containing this acid should be used as the liberated chlorine anions might injure the anode.

The electrodes are attached on both sides to the couplings and supports by wire made of the same steel. Wire and electrode must be electrically welded. The electric current is fed through the centre of the knobs and the axle of the coupling which assures a ready change of polarity after each run and a safe contact between electrode and electric pole.

### *Stretching of the strips*

The vessels are filled with buffer solution and the electrodes are put in place. The dry ends of the paper strips are inserted into the slots, care being taken that the starting line and the polarity of the electrodes are in agreement. After the entire length of the strips has become soaked with buffer from both vessels one can proceed to stretch them. One of the vessels, *e.g.* the right hand one is turned by knob 30° towards the middle of the tank while the other remains fixed in position by its coupling. One must then wait until an equilibrium between the suction of the buffer at both ends of the strip is reached (with a Macherey-Nagel filter No. 61 this takes about 60 minutes[3]). Then the vessel is turned back by knob until resistance is felt. The strips are now sufficiently stretched and the runs can be started by depositing drops of the solution being investigated on the starting line in the normal way.

*Department of Parasitology and Parasitic Diseases, High School of Agriculture, Wroclaw and Department of Animal Physiology, Zoological Institute, University of Wroclaw (Poland)*

ADAM SENIÓW

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Received December 9th, 1959

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* The use of this steel was suggested by Professor E. ZUBIK.

# Effect of an argon-nitrogen carrier gas mixture on the sensitivity of a gas chromatographic ionisation detector

The Pye Argon Gas–Liquid Chromatograph is easily modified for work on a preparative scale. The main problem was how to control the highly sensitive detector; an overloaded detector will give a full scale deflection together with a series of false peaks where there should be only one peak ("peak doubling"). We needed to adapt the detector to give nearly full scale deflections on the recorder for fractions ranging from micrograms to 1 g.

By using a carrier gas mixture of argon and nitrogen we can vary the sensitivity of an argon ionisation detector continuously between the limits obtained with pure argon and pure nitrogen. The detector will give full scale recorder readings without overloading or "peak doubling" for sample fractions varying in size from micrograms to over 1 g. Any user of a Pye Argon Chromatograph should be able to convert the instrument for this type of versatile preparative scale working.

### *Methods of increasing detector sensitivity range*

We wished to avoid the complication of using a by-pass system, and we considered the following three methods for increasing the sensitivity range of the detector.

(1) Decreasing the detector voltage below 750 V. Lowering the detector voltage to about 250 V and 50 V lowers the amplitude of the peaks, but it does not stop "peak doubling".

(2) Using nitrogen as the carrier gas and altering the recorder range from 0–10 mV to 0–1 mV. Pure nitrogen reduces the sensitivity of the detector, but then sample fractions of hundreds of milligrams are needed to get a reasonable response on the recorder. This would only satisfy our requirements for the largest fractions, and we would be near the limit for overloading the column. The apparent sensitivity can be increased by altering the range of the recorder from 0–10 mV to 0–1 mV, but this increase is not sufficient and it is spoiled by a high noise level.

(3) Using a carrier gas mixture of argon and nitrogen. This method gives the detector the sensitivity range that we require. By altering the percentage of nitrogen in the mixture, the detector can be made to work at any sensitivity between those obtained with pure argon and pure nitrogen. This was selected as the only suitable method.

### *Modification and operation of the Pye Argon Chromatograph*

Three stainless steel columns (4 ft. × 2 cm diam.) joined by steel capillary U-tubes are housed in a pipe (2½ in. diam.) which fits within the usual heater jacket. This pipe is packed with aluminium powder which forms the heat conductor. A Variac transformer controls the heat input to the column, vapouriser and outlet tube, which are not controlled by thermostats.

The two carrier gases are controlled by separate needle valves and rotameters before being mixed at a glass Y-junction and passed through a final rotameter. Both gases are kept at the same pressure at the cylinder heads. As we use the rotameters under different conditions from those for which they were calibrated, all our nitrogen percentage values are nominal.

The column was packed with 20 % M. S 710 silicone oil on Embacel (60/100 mesh). The total flow rate was nominally 800 ml/min, the inlet pressure was 25 lb./sq. in., the temperature was 90° and the detector voltage was 2000 V.

*Results*

The same experimental conditions apply to the results shown in Figs. 1 and 2.

Fig. 1 shows the effect of varying the nominal nitrogen percentage from 1 % to 100 % on a chromatogram of 50 mg of impure *n*-pentanol. The impurities help to show up the differences more clearly. Pure argon gives a straight line along the top of the chart and this is not shown on Fig. 1.

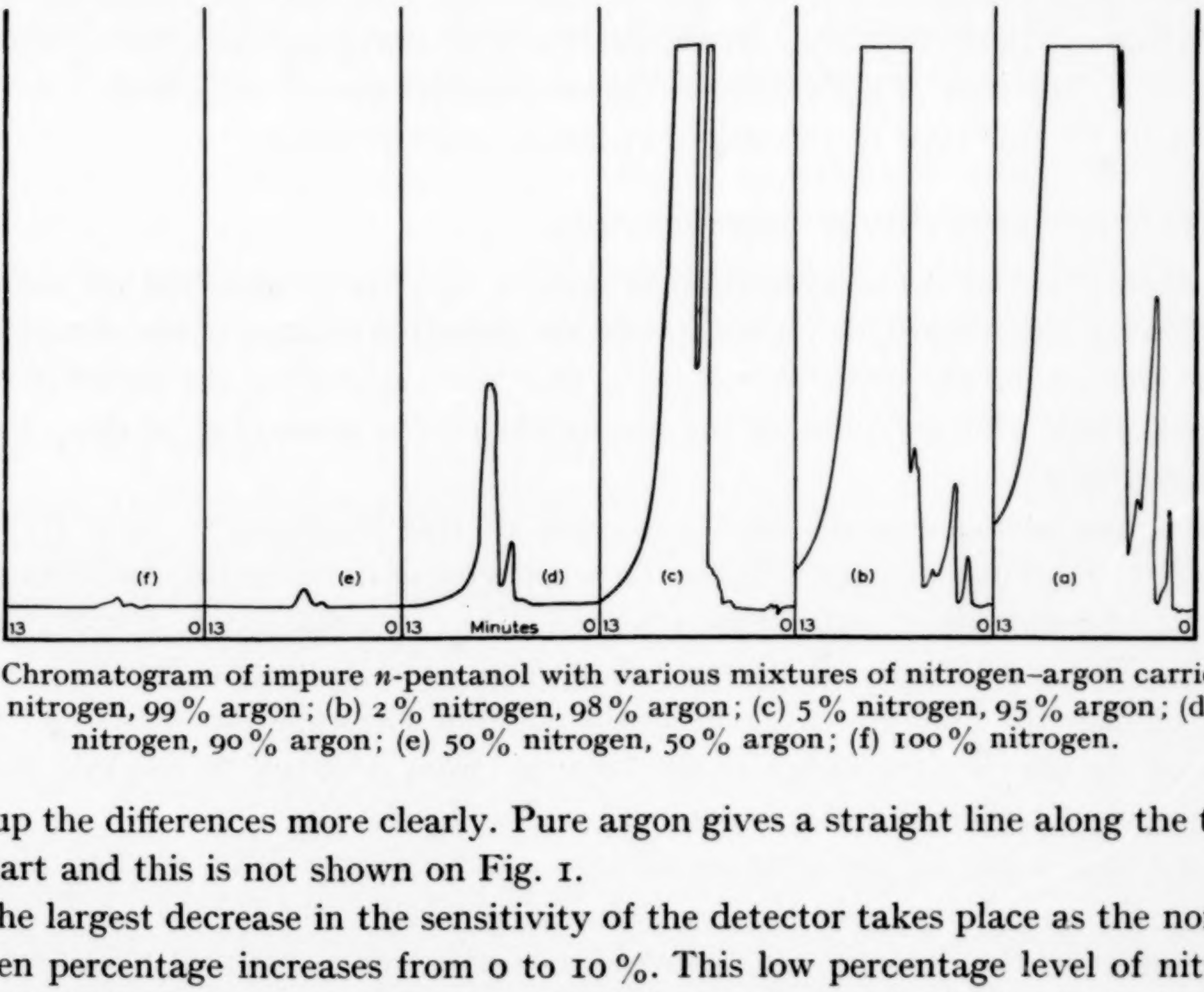

Fig. 1. Chromatogram of impure *n*-pentanol with various mixtures of nitrogen–argon carrier gas. (a) 1 % nitrogen, 99 % argon; (b) 2 % nitrogen, 98 % argon; (c) 5 % nitrogen, 95 % argon; (d) 10 % nitrogen, 90 % argon; (e) 50 % nitrogen, 50 % argon; (f) 100 % nitrogen.

The largest decrease in the sensitivity of the detector takes place as the nominal nitrogen percentage increases from 0 to 10 %. This low percentage level of nitrogen means that the flow-rate settings are critical for good reproducibility. It is therefore better to use the highest possible nitrogen percentage. This is done by adjusting the two main variables that effect the sample vapour concentration in the detector, *i.e.* flow rate and temperature. The flow rate must be as high and the temperature as low as possible.

Although the detector voltage has no control over peak doubling, it does affect the amplitude of both single and double peaks. Provided that doubling has been avoided, the detector voltage can be used in conjunction with the nitrogen percentage, flow rate and temperature to give the optimum control over the detector sensitivity.

The maximum and minimum sample sizes are detected when the carrier gas is either pure nitrogen or pure argon. Using nitrogen and overloading the column, we can deal with more than 1 g of a single substance. Using a nominal 5 % nitrogen in argon as an example of a mixture, our limits of detection of a single substance are about 0.5 mg to 100 mg under the experimental conditions given for Figs. 1 and 2. The exact amount increases with the retention time.

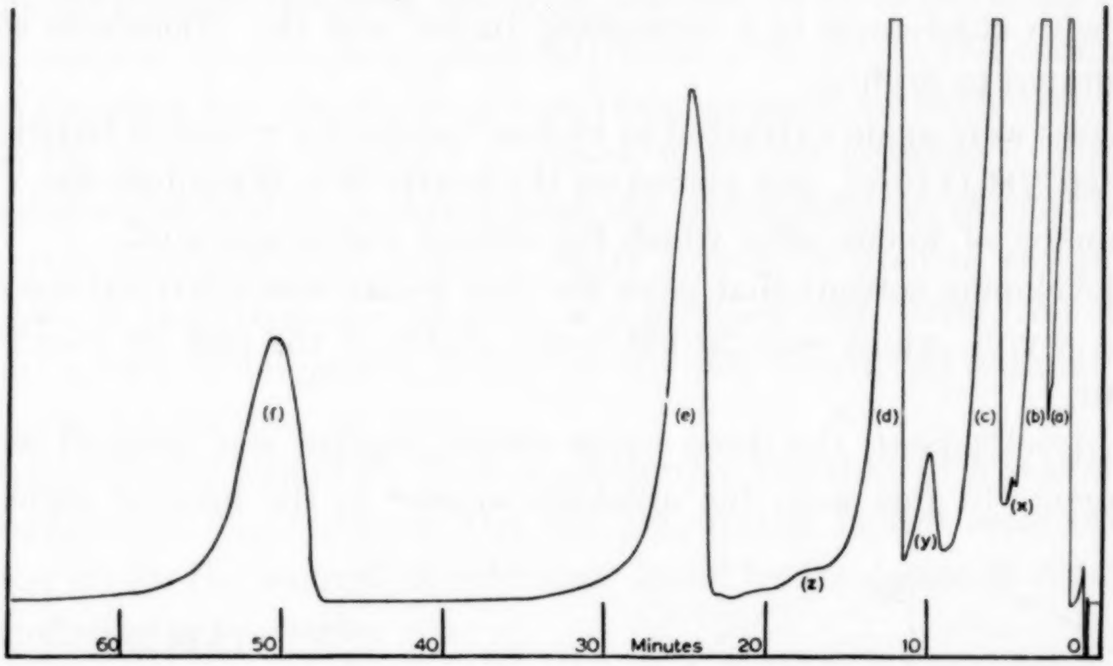

Fig. 2. Chromatogram of a 110 mg mixture of normal alcohols. (a) *n*-propanol; (b) *n*-butanol; (c) *n*-pentanol; (d) *n*-hexanol; (e) *n*-heptanol; (f) *n*-octanol; (x), (y) and (z) impurities.

Fig. 2 shows a separation of a 110 mg mixture of equal quantities of *n*-propanol, *n*-butanol, *n*-pentanol, *n*-hexanol, *n*-heptanol and *n*-octanol. The carrier gas contained a nominal 5 % of nitrogen in argon.

We wish to thank the directors of Unilever Ltd. for permission to publish this note.

*Unilever Ltd., Food Research Department, Colworth House,* D. WELTI
*Sharnbrook, Bedford (Great Britain)* T. WILKINS

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# Paper chromatography of *Withania somnifera* alkaloids

Although *Withania somnifera* have been previously studied[1–3] no simple method has been reported in the literature for the detection and separation of its alkaloidal components.

In a previous paper we already showed the possibility of this separation, by employing a circular chromatographic technique, with which at least five of the alkaloidal components could be detected[4].

On the basis of these results and by employing paper disks with suitable diameter, we succeeded in separating and observing all eight components at the same time.

With our technique it is sufficient to work with 50 g of powdered roots which is the part of the plant richest in alkaloids.

The roots were extracted by percolation with distilled water, until the test with Dragendorff's reagent was negative. The brownish percolate was concentrated and adjusted with aqueous ammonium to a high alkaline pH value. The free bases were extracted with chloroform in a separating funnel and the chloroform solution was then evaporated to dryness.

The bases were again extracted as hydrochlorides by means of butanol saturated with 10% $HCl/H_2O$ (v/v), and placed on the centre of a Whatman No. 1 paper disk with a diameter of 30 cm, after which the solvent was evaporated.

The developing solvent that gave the best results was water-saturated butanol–ethanol (95:5 v/v), which was carried to the centre of the disk by means of a clean wool thread.

After development, the dried paper chromatogram was sprayed with Dragendorff's reagent. In this way, the alkaloids appear in the form of eight concentric rings with red colours of varying intensity on a pale orange background. A map of this chromatogram, is shown in Fig. 1. The $R_F$ values are given in Table I.

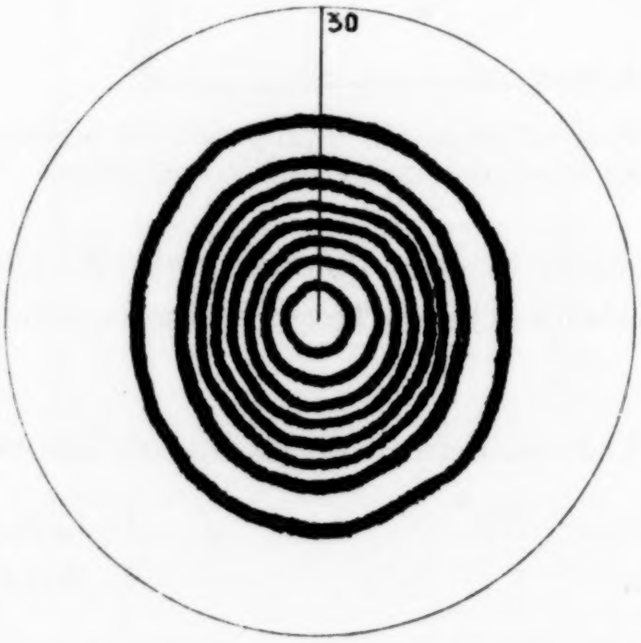

Fig. 1. Circular chromatogram of the eight alkaloidal components.

TABLE I

| *Hydrochloride of alkaloid* | $R_F$ |
|---|---|
| I | 0.67 |
| II | 0.49 |
| III | 0.40 |
| IV | 0.35 |
| V | 0.30 |
| VI | 0.26 |
| VII | 0.20 |
| VIII | 0.10 |

Further studies indicated that this very simple technique can be employed for the separation and detection of the alkaloidal constituents of other plant extracts.

*Institute of Pharmaceutical Chemistry and Toxicology,* MARIO COVELLO
*University of Naples (Italy)* GIUSEPPE CIAMPA

[1] F. B. POWER AND A. H. SALVAY, *J. Chem. Soc.*, 99 (1911) 490.
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## BOOK REVIEW

*Analyst's Pocket-Book*, by J. R. MAJER, Butterworths Scientific Publications, London, 1959, ix + 100 pages, price 17 s. 6 d.

According to the preface, this book has been produced to serve the needs of students and routine analysts by providing quantitative data "in simple and compact form". The form is compact but the simplicity at times appears to have been overdone; especially in the section on standard solutions, where for example it is pointed out that basic solutions should be protected from the atmosphere but no mention is made of the fact that carbonate-free distilled water should be used in the preparation.

The tables of indicators are conveniently grouped and useful, but it is a pity that the more recent chelatometric indicators are only mentioned without giving the conditions under which they are used.

Approximately one half of the book is taken up with tables of gravimetric factors and their logarithms. One wonders why the gravimetric factors were computed so that one must divide the amount of substance found by the factor in order to obtain the amount of substance sought.

It is rather distressing to see that the practice of tabulating densities of solutions at 15° C, without giving temperature coefficients has been continued in yet another book. An otherwise handy set of tables loses a lot of its usefulness, for few laboratories nowadays have an ambient temperature of 15° C.

Although the needs of students are adequately covered, the practising analyst would find that his needs are not so well covered.

Typographical errors are few and the presentation is good.

T. J. BECKMANN (Brisbane)

*J. Chromatog.*, 3 (1960) 593

## ERRATUM

*J. Chromatog.*, Vol. 2 (1959), page 655

In the short communication "The analysis of isoprene by gas chromatography", by F. ARMITAGE, the wrong correction factor for the pressure gradient along the column was used.

The factor used was:

$$\frac{3\,(p_i/p_o)^2 - 1}{2\,(p_i/p_o)^3 - 1}$$

which should have been:

$$\frac{3}{2} \cdot \frac{(p_i/p_o)^2 - 1}{(p_i/p_o)^3 - 1}$$

The amended correction factors and partition coefficients are given below.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Table I: | $f$ | 0.7368 | 0.7392 | 0.6261 | 0.6322 | 0.7481 | 0.7526 |
| | $K$ for isoprene | 68.86 | 67.57 | 105.4 | 106.3 | 134.9 | 137.5 |
| Table II: | $f$ | 0.8167 | 0.8182 | 0.7185 | 0.7245 | 0.7291 | 0.7306 |
| | $K$ for isoprene | 82.07 | 79.70 | 109.2 | 108.1 | 143.4 | 142.6 |

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## TABLE 1

### $R_F$ VALUES OF SOME ALKALOIDS

(D. WALDI, *Arch. Pharm.*, 292 (1959) 206)

Paper: Schleicher & Schüll 2043 b Mgl impregnated with formamide–acetone (2:8) and then pressed. No equilibration.

Solvents: I = Petroleum–diethylamine (9:1)
II = Cyclohexane–diethylamine (9:1)
III = Cyclohexane–chloroform–diethylamine (7:2:1)
IV = Cyclohexane–chloroform–diethylamine (5:4:1)
V = Cyclohexane–chloroform–diethylamine (3:6:1)
VI = Cyclohexane–chloroform–diethylamine (1:8:1)
VII = Chloroform–diethylamine (9:1).

$$R_F \text{ corr.} = \frac{R_F \text{ substance (found)}}{R_F \text{ standard (found)}} \times R_F \text{ standard (average value).}$$

| *Alkaloid* | *$R_F$ corr.* | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Codeine as standard* | | | | *Dihydromorphinone as standard* | | |
| | *I* | *II* | *III* | *IV* | *V* | *VI* | *VII* |
| *Alkaloids of the aromatic amine type* | | | | | | | |
| Amphetamine | 0.47 | 0.58 | 0.86 | 1.0 | 1.0 | 1.0 | 1.0 |
| Ephedrine | 0.26 | 0.47 | 0.76 | 0.87 | 0.95 | 0.96 | 1.0 |
| Hordenine | 0.11 | 0.27 | 0.60 | 0.83 | 1.0 | 1.0 | 1.0 |
| Cathine | 0.06 | 0.22 | 0.71 | 0.71 | 0.91 | 1.0 | 1.0 |
| Capsaicin | 0.04 | 0.10 | 0.77 | 1.0 | 1.0 | 1.0 | 1.0 |
| *Alkaloids with a pyrrole-pyridine ring system* | | | | | | | |
| Sparteine | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Apoatropine | 0.92 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| New psicaine | 0.89 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Tropacocaine | 0.81 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Nicotine | 0.78 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Cocaine | 0.70 | 0.71 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Procaine | 0.17 | 0.25 | 0.67 | 0.89 | 1.0 | 1.0 | 1.0 |
| Homatropine | 0.11 | 0.27 | 0.75 | 0.95 | 1.0 | 1.0 | 1.0 |
| Atropine | 0.10 | 0.33 | 0.78 | 0.95 | 1.0 | 1.0 | 1.0 |
| Scopoline | 0.07 | 0.11 | 0.46 | 0.77 | 1.0 | 1.0 | 1.0 |
| Scopolamine | 0.03 | 0.12 | 0.54 | 0.85 | 1.0 | 1.0 | 1.0 |
| *Alkaloids with a quinoline ring* | | | | | | | |
| Cinchonine | 0.23 | 0.51 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Quinidine | 0.22 | 0.47 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Quinine | 0.19 | 0.40 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Cupreine | 0.0 | 0.0 | 0.12 | 0.31 | 0.58 | 0.73 | 0.77 |
| *Alkaloids with an isoquinoline ring* | | | | | | | |
| Emetine | 0.25 | 0.37 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Bulbocapnine | 0.13 | 0.13 | 0.76 | 0.94 | 1.0 | 1.0 | 1.0 |
| Cephaeline | 0.04 | 0.05 | 0.93 | 1.0 | 1.0 | 1.0 | 1.0 |
| Apomorphine | 0.0 | 0.0 | 0.27 | 0.96 | 1.0 | 1.0 | 1.0 |
| Berberine | 0.0 | 0.04 | 0.13 | 0.30 | 0.46 | 0.77 | 0.82 |
| Hydrastinine | 0.0 | 0.01 | 0.12 | 0.33 | 0.68 | 0.83 | 0.88 |
| Boldine | 0.0 | 0.0 | 0.08 | 0.37 | 0.75 | 0.75 | 0.87 |

*(Continued on p. D2)*

TABLE 1 (*continued*)

| *Alkaloid* | *$R_F$ corr.* | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Codeine as standard* | | | | *Dihydromorphinone as standard* | | |
| | *I* | *II* | *III* | *IV* | *V* | *VI* | *VII* |
| *Opium alkaloids* | | | | | | | |
| Eupaverine | 0.85 | 0.95 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Thebaine | 0.31 | 0.35 | 0.83 | 0.95 | 1.0 | 1.0 | 1.0 |
| Narcotine | 0.24 | 0.38 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Ethylmorphine | 0.17 | 0.28 | 0.85 | 0.96 | 1.0 | 1.0 | 1.0 |
| Eucodal | 0.17 | 0.23 | 0.91 | 1.0 | 1.0 | 1.0 | 1.0 |
| Dihydrocodeine | 0.09 | 0.20 | 0.65 | 0.90 | 1.0 | 1.0 | 1.0 |
| Codeine | 0.06 | 0.13 | 0.56 | 0.84 | 1.0 | 1.0 | 1.0 |
| Papaverine | 0.07 | 0.09 | 0.89 | 0.95 | 1.0 | 1.0 | 1.0 |
| Dihydrocodeinone | 0.05 | 0.09 | 0.54 | 0.90 | 1.0 | 1.0 | 1.0 |
| Dihydromorphinone | 0.0 | 0.03 | 0.10 | 0.28 | 0.72 | 0.87 | 0.86 |
| Cotarnine | 0.0 | 0.03 | 0.14 | 0.40 | 0.65 | 0.90 | 1.0 |
| Morphine | 0.0 | 0.01 | 0.05 | 0.15 | 0.37 | 0.54 | 0.62 |
| Narceine | 0.0 | 0.0 | 0.0 | 0.02 | 0.03 | 0.18 | 0.42 |
| *Rauwolfia alkaloids* | | | | | | | |
| Raubasine | 0.73 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Reserpinine | 0.72 | 0.95 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Rauwolscine | 0.20 | 0.43 | 0.80 | 0.87 | 1.0 | 1.0 | 1.0 |
| Reserpine | 0.10 | 0.22 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Ajmaline | 0.09 | 0.21 | 0.60 | 0.76 | 1.0 | 1.0 | 1.0 |
| Serpentinine | 0.0 | 0.0 | 0.37 | 1.0 | 1.0 | 1.0 | 1.0 |
| Serpentine | 0.0 | 0.0 | 0.01 | 0.09 | 0.51 | 0.81 | 0.84 |
| Neoajmaline | 0.0 | 0.0 | 0.0 | 0.04 | 0.13 | 0.29 | 0.34 |
| Sarpagine | 0.0 | 0.0 | 0.0 | 0.01 | 0.12 | 0.27 | 0.29 |
| *Alkaloids with an indole ring* | | | | | | | |
| Aspidospermine | 0.71 | 0.95 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Yohimbine | 0.07 | 0.19 | 0.56 | 0.85 | 1.0 | 1.0 | 1.0 |
| Strychnine | 0.09 | 0.09 | 0.65 | 0.93 | 1.0 | 1.0 | 1.0 |
| Physostigmine | 0.04 | 0.08 | 0.80 | 0.97 | 1.0 | 1.0 | 1.0 |
| Brucine | 0.02 | 0.02 | 0.53 | 0.93 | 1.0 | 1.0 | 1.0 |
| *Ergot alkaloids* | | | | | | | |
| Ergocristinine | 0.0 | 0.04 | 0.87 | 1.0 | 1.0 | 1.0 | 1.0 |
| Ergocristine | 0.0 | 0.05 | 0.75 | 1.0 | 1.0 | 1.0 | 1.0 |
| Dihydroergocristine | 0.0 | 0.0 | 0.35 | 0.90 | 1.0 | 1.0 | 1.0 |
| Ergotaminine | 0.0 | 0.0 | 0.28 | 0.88 | 1.0 | 1.0 | 1.0 |
| Ergotamine | 0.0 | 0.0 | 0.22 | 0.66 | 1.0 | 1.0 | 1.0 |
| Ergometrinine | 0.0 | 0.0 | 0.19 | 0.54 | 0.86 | 0.91 | 0.91 |
| Dihydroergotamine | 0.0 | 0.0 | 0.08 | 0.42 | 0.95 | 1.0 | 1.0 |
| Ergometrine | 0.0 | 0.0 | 0.02 | 0.08 | 0.25 | 0.45 | 0.53 |
| *Miscellaneous alkaloids* | | | | | | | |
| Cevadine | 0.64 | 0.84 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Aconitine | 0.28 | 0.54 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Gelseminine | 0.08 | 0.26 | 0.70 | 1.0 | 1.0 | 1.0 | 1.0 |
| Veratridine | 0.02 | 0.03 | 0.19 | 0.59 | 0.83 | 0.84 | 0.84 |
| Colchicine | 0.0 | 0.0 | 0.04 | 0.44 | 0.80 | 0.94 | 0.96 |
| Pilocarpine | 0.0 | 0.0 | 0.02 | 0.18 | 0.55 | 0.79 | 0.82 |
| Solanine | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

## TABLE 2

### $R_F$ VALUES OF SOME NON-POLAR ALKALOIDS

(D. WALDI, *Arch. Pharm.*, 292 (1959) 206)

Paper: Schleicher & Schüll 2043 b Mgl impregnated with petroleum.
Solvents: A = Propanol–diethylamine–water (1:1:8)
B = Propanol–diethylamine–water (1.33:1:7.66)
C = Propanol–diethylamine–water (1.66:1:7.33)
D = Propanol–diethylamine–water (2:1:7).

| *Alkaloid* | $R_F$ | | | |
|---|---|---|---|---|
| | *A* | *B* | *C* | *D* |
| Sparteine | 0.03 | 0.07 | 0.08 | 0.22 |
| Psicaine | 0.10 | 0.18 | 0.36 | 0.53 |
| Eupaverine | 0.15 | 0.25 | 0.46 | 0.84 |
| Tropacocaine | 0.21 | 0.31 | 0.44 | 0.58 |
| Apoatropine | 0.16 | 0.23 | 0.47 | 0.62 |

## TABLE 3

### $R_F$ VALUES OF ORGANIC PEROXIDES

(N. A. MILAS AND I. BELIČ, *J. Am. Chem. Soc.*, 81 (1959) 3358)

Paper: Whatman No. 1.
Solvent systems: 1. N,N-Dimethylformamide–decalin.
2. N-Methylformamide–decalin.
3. *n*-Butanol–ethanol–water (45:5:50).

| *Peroxide* | $R_F$ | | |
|---|---|---|---|
| | *1* | *2* | *3* |
| Hydrogen peroxide | 0.0 | 0.0 | 0.49 |
| *p,p'*-Dicyanobenzoyl peroxide | 0.0 | 0.01 | 0.0–0.50 |
| *m*-Monocyanobenzoyl peroxide | 0.20 | 0.03 | 0.97 |
| *m*-Monomethoxybenzoyl peroxide | 0.29 | 0.30 | 0.94 |
| Benzoyl peroxide | 0.38 | 0.37 | 0.94 |
| *p,p'*-Dichlorobenzoyl peroxide | 0.56 | 0.40 | 0.94 |
| 2,4-Dichlorobenzoyl peroxide | 0.69 | 0.44 | 0.94 |
| 2,5-Dimethylhexane 2,5-dihydroperoxide | 0.00 | 0.18 | 0.89 |
| 2,5-Dimethyl-3-hexyne 2,5-dihydroperoxide | 0.00 | 0.17 | 0.89 |
| 2,5-Dimethyl-3-hexyne 2,5-diperbenzoate | 0.29 | 0.24 | 0.97 |
| 2,7-Dimethyl-3,5-octadiyne 2,7-dihydroperoxide | 0.00 | 0.10 | 0.92 |
| Bis-(1-hydroxyheptyl) peroxide | 0.00 | 0.40 | |
| *tert*.-Butyl hydroperoxide | 0.00 | 0.00 | |
| Cumene hydroperoxide | 0.09 | 0.30 | 0.91 |
| *tert*.-Butylperoxymaleic acid | 0.00 | 0.10 | 0.38 |
| *tert*.-Butyl perbenzoate | 0.63 | 0.54 | 0.95 |
| Lauroyl peroxide | 0.98 | 0.88 | 0.97 |
| 3,3-Dihydroperoxy-pentane | 0.02 | | |
| 3,3'-Dihydroperoxy-3,3'-dipentylperoxide | 0.53 | | |
| 1,1,4,4,7,7,10,10-Octaethyl-1,4,7-triperoxy-1,10-dihydroperoxide | 0.86 | | |
| 1,1,4,4,7,7-Hexaethyl-1,4,7-cyclononatriperoxane | 0.91 | | |

## TABLE 4

### $R_F$ VALUES OF QUATERNARY AMMONIUM SALTS

(G. B. Marini-Bettòlo and M. Miranda, *Rend. ist. super. sanità*, 17 (1954) 463)

Solvent: butanol–acetic acid–water (4.3:1.4:4.3).
Paper: Whatman No. 1.

| *Compound* | *$R_F$ value* |
|---|---|
| $CH_3CH_2CH_2N(CH_3)_3I$ | 0.67 |
| $CH_2CH_2CH_2ON(CH_3)_3I$ | 0.70 |
| $CH_3ON(CH_3)_3I$ | 0.53 |
| $CH_3N(CH_3)_3Br$ | 0.43 |
| $C_2H_5N(C_2H_5)_3I$ | 0.61 |
| $C_2H_5N(CH_3)_3I$ | 0.52 |
| $C_2H_5ON(CH_3)_3I$ | 0.64 |
| Flaxedil | 0.40 |
| Succinylcholine iodide | 0.39 |
| Methylamine hydrochloride | 0.52 |

## TABLE 5

### $R_F$ VALUES OF SUGAR PHENYLOSAZONES AS THEIR BORATE COMPLEXES

(B. Arreguin, *Anal. Chem.*, 31 (1959) 1371)

Paper: Schleicher and Schüll 2040 impregnated with a 0.1 *M* solution of potassium tetraborate at pH 9.3 and dried at 60°.
Technique: descending development.
$R_X = R_F$ taking xylose as 1.
Solvents: A. 75% dioxane–hexane–water (60:60:30 v/v).
B. 75% dioxane–hexane–water–benzene (55:50:25:20 v/v).
C. 75% dioxane–water–1,2-dichloroethane (54:40:6 v/v).

| *Osazone of* | *$R_X$* | *$R_F$* | |
|---|---|---|---|
| | *A* 21° | *B* 21° | *C* 17° |
| Arabinose | 1.80–1.90 | 0.65–0.65 | 0.92–0.93 |
| Xylose | 1.00–1.00 | 0.28–0.30 | 0.75–0.77 |
| Glucose | 0.40–0.41 | 0.07–0.08 | 0.30–0.34 |
| Fructose | 0.40–0.42 | 0.08 | 0.31–0.33 |
| Rhamnose | 1.20–1.28 | 0.36–0.37 | 0.82–0.83 |
| Galactose | 0.53–0.55 | 0.12–0.13 | 0.54–0.55 |
| Sorbose | 0.43–0.45 | 0.09–0.09 | 0.37–0.40 |
| Cellobiose | 0.14–0.15 | 0.02–0.05 | 0.01 |
| Melibiose | 0.06–0.07 | 0.05–0.06 | 0.04 |
| Maltose | 0.04–0.08 | 0.04–0.05 | 0.01 |
| Lactose | 0.31–0.34 | 0.05–0.05 | 0.06–0.09 |

## ERRATUM

*Chromatog. Data*, 2 (1959) D 28, D 29.

Table 47, heading of the second column: for *β,β′-Hydroxydipropionitrile* read *β,β′-Oxydipropionitrile.*

## TABLE 6

### $R_F$ VALUES OF SOME INDOLE DERIVATIVES

(Ž. Procházka, V. Šanda and K. Macek (*Collection Czechoslov. Chem. Communs.*,24 (1959) 2928)

Solvents: (a) Ethyl acetate.
(b) Ethyl butyrate.
(c) Butyl butyrate.
(d) Di-*n*-butyl ether.
(e) Di-amyl ether.

Paper: Whatman No. 4 (ascending).

Detection: Formaldehyde reagent, Ehrlich reagent, Salkowski reagent, 2,4-dinitrophenylhydrazone reagent, Jaffe reagent.

| *Compound* | *Solvent* | | | | |
|---|---|---|---|---|---|
| | *(a)* | *(b)* | *(c)* | *(d)* | *(e)* |
| Ascorbigen | 0.82 | 0.31 | 0.14 | Start | Start |
| 3-(3-Indolyl)-propandiol-1,2 | 0.85 | 0.61 | 0.51 | 0.08 | Start |
| Indole-3-carboxylic acid | 0.90 | 0.83 | 0.81 | 0.43 | 0.14 |
| Indole-3-aldehyde | 0.93 | 0.89 | 0.86 | 0.58 | 0.32 |
| 3-Indolyl-acetic acid | 0.96 | 0.87 | 0.86 | 0.57 | Streaking from start |
| 3-(3-Indolyl)-butyric acid | Front | Front | Front | 0.82 | 0.44 |

## TABLE 7

### $R_F$ VALUES OF ANTHRANILIC ACID AND SOME SYNTHETIC AMADORI COMPOUNDS DERIVED FROM CERTAIN N-GLYCOSYLAMINES OF ANTHRANILIC ACID

(C. H. Doy and F. Gibson, *Biochem. J.*, 72 (1959) 586)

Solvent: Methanol–butan-1-ol–benzene–water (2:1:1:1, by vol.).

Length of run: 19 cm (ascending).

Paper: Whatman No. 1.

Detection: U.V. fluorescence, and where suitable ammonium molybdate, aniline acetate, phloroglucinol, anthrone and alkaline 2,3,5-triphenyltetrazolium chloride.

| *Compound* | *Parent glycosylamine* | $R_F$ |
|---|---|---|
| Anthranilic acid | — | 0.93 |
| 1-(*o*-Carboxyphenylamino)-1-deoxy-D-ribulose | N-*o*-Carboxyphenyl-D-arabinosylamine | 0.86 |
| | N-*o*-Carboxyphenyl-D-ribosylamine | 0.85 |
| 1-(*o*-Carboxyphenylamino)-1-deoxy-L-ribulose | N-*o*-Carboxyphenyl-L-arabinosylamine | 0.85 |
| 1-(*o*-Carboxyphenylamino)-1-deoxy-D-xylulose | N-*o*-Carboxyphenyl-D-xylosylamine | 0.84* |
| 1-(*o*-Carboxyphenylamino)-1-deoxy-D-fructose | N-*o*-Carboxyphenyl-D-glucosylamine | 0.80 |

* The small difference between the xylulose and ribulose compounds was confirmed on many chromatograms.

## TABLE 8

### $R_F$ VALUES OF FAT-SOLUBLE DYES

(M. R. VERMA AND RAMJI DAS, *J. Sci. Ind. Research (India)*, 16B (1957) 131)

Paper: Whatman No. 1.
Technique: ascending.
Temperature 28–30°.

| Dye | *$R_F$ value on filter paper impregnated with* | | | | | |
|---|---|---|---|---|---|---|
| | *Liquid paraffin, using eluant* | | | | *Lauryl alcohol,* | |
| | *Water–acetone (30:70)* | *Water–dioxane (40:60)* | *Water–methyl alcohol (34:66)* | *Ethylene glycol* | *Water–acetone (35:65)* | *Water–dioxane (45:55)* |
| *Yellow* | | | | | | |
| 1 Fat Yellow S.G. (Ciba) | 0.00 | 0.00 | 0.00 | 0.00 | 00.0 | 0.00 |
| 2 Waxoline Yellow A.D.S. (I.C.I.) | 0.45 | 0.40 | 0.22 | 0.40 | 0.27 | 0.21 |
| 3 F.D. & C. Yellow No. 4 (F.D.A.) | 0.50 | 0.43 | 0.16 | 0.57 | 0.16 | 0.12 |
| 4 F.D. & C. Yellow No. 3 (F.D.A.) | 0.58 | Spot vanished | 0.28 | 0.71 | 0.21 | 0.15 |
| 5 Spirit Yellow (Synth.) | 0.77 | 0.71 | 0.61 | 0.90 | 0.32 | 0.26 |
| 6 Organol Yellow A.N.P. (French) | 0.86 | 0.81 | 0.70 | 0.90 | 0.25 | 0.20 |
| 7 Ceres Orange G (Chika) | Tailing | Tailing | Tailing | Spot vanished | 0.21 | Tailing |
| 8 Oil Yellow B (Synth.) | 0.93 | 0.86 | 0.70 | 0.95 | 0.52 | 0.43 |
| 9 Martius Yellow (B.D.H.) | 0.98 | 0.94 | 0.81 | 1.00 | 1.00 | 0.90 |
| 10 Chrysoidine (Synth.) | 0.91 | 0.88 | Fades | Spot vanished | 0.62 | 0.56 |
| *Orange* | | | | | | |
| 11 F.D. & C. Orange No. 2 (F.D.A.) | 0.22 | 0.23 | 0.06 | 0.16 | 0.17 | 0.11 |
| 12 Fat Orange 4A (Ciba) | 0.29 | 0.30 | 0.09 | 0.28 | 0.21 | 0.13 |
| *Red* | | | | | | |
| 13 F.D. & C. Red No. 32 | 0.16 | 0.18 | 0.03 | 0.08 | 0.12 | 0.07 |
| 14 Fat Red 7B (Ciba) | 0.11 | 0.13 | 0.00 | 0.00 | 0.10 | 0.03 |
| 15 Red Powder H 4887 (Bush) | 0.21 | 0.26 | 0.03 | 0.13 | 0.15 | 0.07 |
| 16 Organol Red BS (French) | 0.45<br>0.11 | 0.46<br>0.13 | 0.13<br>0.00 | 0.33<br>0.00 | 0.23<br>0.10 | 0.16<br>0.03 |
| 17 Fat Red B (Ciba) | 0.40 | 0.46 | 0.12 | 0.29 | 0.15 | 0.10 |
| 18 Ceres Red G (Chika) | 0.50 | 0.56 | 0.18 | 0.49 | 0.28 | 0.20 |
| 19 Fast Pink B (Amritlal) | 0.96 | 0.96 | 0.87 | 1.00 | 0.83 | 0.86 |
| *Brown* | | | | | | |
| 20 Fat Brown 3R (Ciba) | 0.64 | 0.72 | 0.33 | Spot vanished | 0.20 | 0.14 |
| 21 Oil Brown 58217 (Amritlal) | 0.85 | 0.85 | 0.43 | 0.70 | 0.33 | 0.31 |
| 22 Oil Brown (Synth.) | 0.72<br>0.15 | 0.72<br>0.23 | 0.33<br>0.00 | Spot vanished<br>0.13 | 0.21<br>0.18 | 0.14<br>0.07 |

Oil Yellow D (Atul), Oil Yellow (Saco) and Sudan Yellow GG (Capco) give same values as 2; Sudan Orange G (Capco) gives same values as 7; Waxoline Yellow O.S. (I.C.I.) gives same value as 8; Chrysoidine B (Capco) gives same value as 10; Oil Orange 33570 (Amritlal), Ceres Orange R (Chika), Oil Orange (Saco), Waxoline Yellow I.S. (I.C.I.), Oil Orange R (Atul) and Sudan Orange

Method of impregnation of paper: the paper sheets were dipped in the following solutions:

(a) Liquid paraffin B.P. in petroleum ether (5 %).
(b) Lauryl alcohol in petroleum ether (1.5 %).
(c) Oleic acid in petroleum ether (2 %).
(d) Diethylene glycol monostearate in petroleum ether (1.5 %).

| *$R_F$ value on filter paper impregnated with* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *using eluant* | | *Oleic acid, using eluant* | | | | *Diethylene glycol monostearate, using eluant* | | | |
| *Water–methyl alcohol (30:70)* | *Water–acetic acid (45:55)* | *Water–acetone (25:75)* | *Water–methyl alcohol (15:85)* | *Water–dioxane (33:67)* | *Ethylene glycol* | *Water–acetone (30:70)* | *Water–methyl alcohol (20:80)* | *Water–dioxane (33:67)* | *Water–ethyl cellosolve (48:52)* |
| 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 0.37 | 0.80 | 0.31 | 0.50 | 0.31 | 0.54 | 0.52 | 0.57 | 0.52 | 0.58 |
| 0.19 | 0.11 | 0.27 | 0.40 | 0.31 | 0.17 | 0.56 | 0.39 | 0.43 | 0.52 |
| 0.27 | 0.15 | 0.29 | 0.45 | 0.47 | 0.17 | 0.39 | 0.47 | 0.52 | 0.52 |
| 0.45 | 0.70 | 0.38 | 0.62 | 0.47 | Spot vanished | 0.56 | 0.64 | 0.62 | 0.63 |
| 0.34 | 0.25 | 0.38 | 0.62 | 0.47 | 0.84 | 0.56 | 0.57 | 0.62 | 0.63 |
| Spot vanished | Spot vanished | Tailing | Spot deformed | Spot vanished | Spot vanished | Tailing | Spot vanished | Spot vanished | Spot vanished |
| 0.59 | 0.80 | 0.49 | 0.70 | 0.58 | 1.00 | 0.68 | 0.66 | 0.74 | 0.68 |
| 0.67 | 0.66 | 1.00 | 0.70 | 1.00 | 1.00 | 1.00 | 0.75 | Spot vanished | 0.85 |
| 0.53 | 0.92 | 0.45 | Spot vanished | 0.82 | Spot vanished | Spot vanished | — | — | — |
| 0.17 | 0.13 | 0.25 | 0.28 | 0.22 | 0.26 | 0.31 | 0.39 | 0.41 | 0.35 |
| 0.24 | 0.21 | 0.32 | 0.34 | 0.26 | 0.36 | 0.38 | 0.47 | 0.45 | 0.49 |
| 0.13 | 0.09 | 0.20 | 0.22 | 0.22 | 0.17 | 0.35 | 0.35 | 0.35 | 0.41 |
| 0.04 | 0.00 | 0.15 | 0.12 | 0.11 | 0.04 | 0.13 | 0.11 | 0.23 | 0.10 |
| 0.14 | 0.05 | 0.26 | 0.21 | 0.20 | 0.20 | 0.21 | 0.33 | 0.39 | 0.31 |
| 0.24 | 0.13 | 0.35 | 0.33 | 0.28 | 0.26 | 0.35 | 0.45 | 0.42 | 0.42 |
| 0.04 | 0.00 | 0.15 | 0.12 | 0.11 | 0.04 | 0.13 | 0.12 | 0.23 | 0.10 |
| 0.22 | 0.25 | 0.28 | 0.31 | 0.23 | 0.22 | 0.30 | 0.45 | 0.42 | 0.30 |
| 0.29 | 0.21 | 0.41 | 0.36 | 0.34 | 0.37 | 0.44 | 0.52 | 0.55 | 0.53 |
| 0.82 | 0.96 | 0.59 | 0.64 | 0.81 | 0.77 | 0.85 | 0.81 | 0.91 | 0.81 |
| 0.20 | 0.53 | 0.36 | 0.41 | 0.34 | 0.58 | 0.32 | 0.36 | 0.47 | 0.34 |
| 0.40 | 0.78 | 0.50 | 0.52 | 0.53 | 0.66 | 0.46 | 0.46 | 0.67 | 0.48 |
| 0.20 | 0.53 | 0.35 | 0.41 | 0.34 | 0.58 | 0.32 | 0.37 | 0.47 | 0.34 |
| 0.16 | 0.05 | 0.26 | 0.21 | 0.20 | 0.13 | 0.19 | 0.33 | 0.39 | 0.31 |

(Capco) give same values as 12; Oil Red (Saco) and Oil Red (Atul) give same values as 14; Waxoline Red B.N.D. (I.C.I.), Sudan G III (Synth.) and Oil Red AB (Atul) give same values as 15; Oil Red G (Atul) and Sudan Red G (Capco) give same values as 18; Oil Brown (Atul) and Sudan Brown B.B. (Capco) give same values as 22.

## TABLE 9

### $R_F$ VALUES OF FOOD COLOURS

(M. R. VERMA AND RAMJI DAS, *J. Sci. Ind. Research* (*India*), 15C (1956) 186)

Paper: Whatman No. 1.
Technique: ascending.
Temperature: 27–32°.
Eluants: A Sodium citrate, 10 g + liquor ammonia, 50 ml; volume made up to 100 ml.
B Sodium butyrate, 6 g + liquor ammonia, 50 ml; volume made up to 100 ml.
C 3% solution of sodium phosphate; pH adjusted to 11.
D Sodium acetate, 3 g + liquor ammonia, 10 ml; volume made up to 100 ml.

| *Name of dye and source* | *$R_F$ values* | | | | |
|---|---|---|---|---|---|
| | *A* | *B* | *C* | *D* | *E* |
| *Yellow dyes* | | | | | |
| Naphthol Yellow S AF (Ciba) | 0.37 | 0.41 | 0.44 | 0.43 | 0.60 |
| Edicol Supra Yellow YS (I.C.I.) | 0.37 | 0.41 | 0.44 | 0.43 | 0.60 |
| F.D. & C. Yellow No. 1 (F.D.A.) | 0.38 | 0.41 | 0.44 | 0.43 | 0.61 |
| Food colour Tartrazine S (Amritlal) | 0.68 | 0.71 | 0.63 | 0.72 | 0.28 |
| Edicol Supra Tartrazine NS (I.C.I.) | 0.68 | 0.71 | 0.63 | 0.72 | 0.27 |
| F.D. & C. Yellow No. 5 (F.D.A.) | 0.68 | 0.71 | 0.63 | 0.72 | 0.27 |
| Edicol Supra Yellow FCS (I.C.I.) | 0.60 | 0.59 | 0.48 | 0.52 | 0.54 |
| F.D. & C. Yellow No. 6 (F.D.A.) | 0.60 | 0.59 | 0.48 | 0.52 | 0.54 |
| Auramine OAF (Ciba) | Spot vanished | 0.39 | Spot vanished | 0.27 | 1.00 |
| Golden Yellow E AF (Ciba) | 0.53 | 0.62 | 0.50 | 0.62 | 0.54 |
| *Orange dyes* | | | | | |
| Hidacid Orange I 21-5005 (Amritlal) | 0.33 | 0.40 | 0.33 | 0.28 | 0.60 |
| F.D. & C. Orange No. 1 (F.D.A.) | 0.33 | 0.40 | 0.33 | 0.28 | 0.61 |
| *Red dyes* | | | | | |
| Ponceaux NRS 19151 (Amritlal) | 0.35 | 0.23 | 0.34 | 0.11 | 0.43 |
| Edicol Supra Ponceaux RS (I.C.I.) | 0.35 | 0.23 | 0.34 | 0.11 | 0.43 |
| F.D. & C. Red No. 1 (F.D.A.) | 0.35 | 0.23 | 0.34 | 0.11 | 0.43 |
| Hidacid Amaranth 21-6008 (Amritlal) | 0.56 | 0.44 | 0.40 | 0.28 | 0.23 |
| Edicol Supra Amaranth AS (I.C.I.) | 0.56 | 0.44 | 0.41 | 0.28 | 0.23 |
| F.D. & C. Red No. 2 (F.D.A.) | 0.56 | 0.44 | 0.40 | 0.28 | 0.23 |
| Edicol Supra Erythrosine AS (I.C.I.) | 0.08 | 0.07 | 0.13 | 0.09 | 0.66 |
| F.D. & C. Red No. 3 (F.D.A.) | 0.08 | 0.07 | 0.13 | 0.09 | 0.66 |
| F.D. & C. Red No. 4 (F.D.A.) | 0.51 | 0.32 | 0.27 | 0.32 | 0.25 |
| Bordeaux Red—RU AF (Ciba) | 0.36 | 0.22 | 0.23 | 0.14 | 0.10 |
| Carmoisine WNN 19153 (Amritlal) | 0.36 | 0.22 | 0.23 | 0.14 | 0.10 |
| Edicol Supra Carmoisine WS (I.C.I.) | 0.36 | 0.22 | 0.23 | 0.14 | 0.10 |
| Food Colour Rose BS (Amritlal) | 0.39 | Tailing | Tailing | 0.23 | 0.95 |
| Edicol Supra Rose BS (I.C.I.) | 0.39 | Tailing | Tailing | 0.23 | 0.95 |
| Edicol Supra Geranine 2 GS (I.C.I.) | 0.62 | 0.54 | 0.44 | 0.42 | 0.30 |
| Edicol Supra Ponceaux 4 RS (I.C.I.) | 0.70 | 0.59 | 0.55 | 0.49 | 0.25 |
| Furnace Red RU AF (Ciba) | 0.34 | 0.34 | 0.29 | 0.17 | 0.53 |
| Edicol Supra Red 10 BS (I.C.I.) | 0.31 | 0.26 | 0.34 | 0.15 | 0.30 |
| *Green dyes* | | | | | |
| Hidacid Emerald Green 21-9005 (Amritlal) | — | — | — | Tailing | 0.83 |
| F.D. & C. Green No. 1 (F.D.A.) | — | — | — | Tailing | 0.82 |
| Hidacid Light Green SF 21-9006 (Amritlal) | | Spot vanished | | 0.83 | 0.58 |
| F.D. & C. Green No. 2 (F.D.A.) | — | — | — | 0.83 | 0.58 |

E Isoamyl alcohol, 40 ml + ethyl alcohol, 40 ml + liquor ammonia, 10 ml + water, 20 ml.
F Acetoacetic ester, 50 ml + methyl alcohol, 20 ml + liquor ammonia, 20 ml.
G (i) 10% sodium citrate solution in water saturated with phenol.
(ii) Sodium citrate, 2 g + phenol, 5 g; volume made up to 100 ml.
H Phenol saturated with 10% solution of sodium citrate.
J Amyl alcohol, 50 ml + methyl alcohol, 25 ml + water, 25 ml.
K *n*-Butyl alcohol, 50 ml + acetic acid (3*N*), 50 ml + ethyl alcohol, 20 ml.
L Isobutyl alcohol, 50 ml + formic acid, 20 ml + water, 25 ml.
M Methyl ethyl ketone, 120 ml + acetic acid (4*N*), 40 ml + water, 25 ml.
N Isopropyl alcohol, 85 ml + water, 15 ml.

The eluants A, B, C, D, E and F are alkaline; G, H, J and N neutral; and K, L and M acidic.

| *$R_F$ values* | | | | | | | |
|---|---|---|---|---|---|---|---|
| *F* | *G* | *H* | *J* | *K* | *L* | *M* | *N* |
| 0.35 | 0.31 | 0.13 | 0.32 | 0.41 | 0.52 | 0.61 | — |
| 0.35 | 0.31 | 0.13 | 0.30 | 0.39 | 0.52 | 0.61 | — |
| 0.35 | 0.32 | 0.12 | 0.30 | 0.39 | 0.52 | 0.62 | — |
| 0.08 | 0.34 | 0.03 | 0.03 | 0.05 | 0.29 | 0.03 | — |
| 0.08 | 0.33 | 0.03 | 0.04 | 0.05 | 0.29 | 0.03 | — |
| 0.08 | 0.33 | 0.03 | 0.04 | 0.05 | 0.29 | 0.03 | — |
| 0.32 | 0.24 | 0.26 | 0.17 | 0.26 | 0.29 | 0.32 | — |
| 0.32 | 0.24 | 0.26 | 0.17 | 0.26 | 0.29 | 0.33 | — |
| 0.88 | 0.00 | 1.00 | 0.86 | 1.00 | 1.00 | 1.00 | — |
| | | | | | | | |
| 0.25 | 0.42 | 0.13 | 0.23 | 0.20 | 0.30 | 0.35 | — |
| | | | | | | | |
| 0.51 | 0.11 | 0.69 | 0.58 | 0.69 | 0.63 | 0.87 | — |
| 0.51 | 0.11 | 0.69 | 0.58 | 0.69 | 0.63 | 0.86 | — |
| | | | | | | | |
| 0.41 | 0.07 | 0.28 | 0.36 | 0.39 | 0.26 | 0.42 | — |
| 0.41 | 0.07 | 0.28 | 0.36 | 0.39 | 0.26 | 0.42 | — |
| 0.41 | 0.07 | 0.28 | 0.36 | 0.39 | 0.26 | 0.40 | — |
| 0.08 | 0.18 | 0.04 | 0.06 | 0.11 | 0.16 | 0.09 | — |
| 0.08 | 0.18 | 0.04 | 0.06 | 0.11 | 0.16 | 0.09 | — |
| 0.08 | 0.18 | 0.04 | 0.06 | 0.11 | 0.16 | 0.09 | — |
| 0.50 | 0.05 | 0.61 | 0.62 | 1.00 | 1.00 | 1.00 | — |
| 0.48 | 0.05 | 0.61 | 0.62 | 1.00 | 1.00 | 1.00 | — |
| 0.31 | 0.11 | 0.39 | 0.25 | 0.39 | 0.05 | 0.43 | — |
| 0.31 | 0.10 | 0.29 | 0.25 | 0.48 | 0.30 | 0.52 | — |
| 0.31 | 0.10 | 0.29 | 0.25 | 0.48 | 0.30 | 0.52 | — |
| 0.31 | 0.10 | 0.29 | 0.25 | 0.47 | 0.30 | 0.52 | — |
| 1.00 | 0.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | — |
| 1.00 | 0.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | — |
| 0.36 | 0.21 | 0.32 | 0.15 | 0.30 | 0.23 | 0.34 | — |
| 0.16 | 0.34 | 0.10 | 0.09 | 0.20 | 0.20 | 0.25 | — |
| 0.58 | 0.10 | 0.70 | 0.65 | 0.74 | 0.66 | 0.82 | — |
| 0.28 | 0.13 | 0.29 | 0.11 | 0.24 | Spot vanished | 0.33 | — |
| | | | | | | | |
| 1.00 | 0.03* | 1.00 | 0.56 | 0.77 | 0.71 | 0.80 | 0.61 |
| 1.00 | 0.03* | 1.00 | 0.56 | 0.77 | 0.71 | 0.80 | 0.61 |
| Fades | 0.37* | 0.54 | 0.34 | 0.46 | 0.34 | Spot vanished | 0.33 |
| Fades | 0.37* | 0.54 | 0.35 | 0.46 | 0.34 | Spot vanished | 0.33 |

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* For footnote see p. D10.

(*continued on pp. D10, D11*)

TABLE 9 (*continued*)

| *Name of dye and source* | *$R_F$ values* | | | | |
|---|---|---|---|---|---|
| | *A* | *B* | *C* | *D* | *E* |
| F.D. & C. Green No. 3 (F.D.A.) | — | — | — | 0.83 | 0.38 |
| Food Colour Green FSL 19122 (Amritlal) | — | — | — | 0.83 | 0.62 (tailing) |
| Edicol Supra Green BS (I.C.I.) | — | — | — | 0.90 | Spot vanished |
| *Blue dyes* | | | | | |
| Food Colour Blue GES 19111 (Amritlal) | — | — | — | 0.70 | 0.62 |
| Edicol Supra Blue EGS (I.C.I.) | — | — | — | 0.70 | 0.62 |
| F.D. & C. Blue No. 1 (F.D.A.) | — | — | — | 0.70 | 0.62 |
| Edicol Supra Blue XS (I.C.I.) | — | — | — | 0.10 | Spot vanished |
| F.D. & C. Blue No. 2 (F.D.A.) | — | — | — | 0.10 | Spot vanished |
| *Violet dyes* | | | | | |
| Violet Powder H 2503 (Bush) | — | — | — | 0.13 | 1.00 |
| F.D. & C. Violet No. 1 (F.D.A.) | — | — | — | 0.13 | 0.82 |

[*] The eluant used was a solution of a mixture of sodium citrate, 2 g and phenol, 5 g in 100 ml.

## TABLE 10

### $R_F$ VALUES OF SOME AMINO ACIDS AT 60°

(J. B. HIMES AND L. D. METCALFE, *Anal. Chem.*, 31 (1959) 1192)

Solvent: Methyl ethyl ketone–propionic acid–water (15:5:6).
Paper: Whatman No. 1 filter paper.
Horizontal development.

| *Amino acid* | *$R_F$* | *Amino acid* | *$R_F$* |
|---|---|---|---|
| Cystine | 0.21 | Alanine | 0.54 |
| Lysine | 0.31 | Proline | 0.58 |
| Histidine | 0.34 | Tyrosine | 0.66 |
| Arginine | 0.38 | Valine | 0.71 |
| Serine | 0.42 | Methionine | 0.73 |
| Aspartic acid | 0.43 | Tryptophan | 0.78 |
| Glycine | 0.46 | Isoleucine | 0.81 |
| Threonine | 0.49 | Phenylalanine | 0.82 |
| Glutamic acid | 0.51 | Leucine | 0.85 |

TABLE 9 (*continued*)

| $R_F$ values | | | | | | | |
|---|---|---|---|---|---|---|---|
| *F* | *G* | *H* | *J* | *K* | *L* | *M* | *N* |
| 0.50 | 0.70 | 0.58 | 0.35 | 0.50 | 0.34 | 0.58 (turns blue) | 0.33 |
| Tailing | 0.37 (tailing) | Tailing | Tailing | Tailing | Tailing | 0.58 | Tailing |
| 0.60 | 0.37 (tailing) | 0.81 | 0.31 | 0.52 | c.50 | 0.72 | 0.44 |
| 0.60 | 0.47 | 0.68 | 0.31 | 0.43 | 0.40 | 0.63 | 0.33 |
| 0.60 | 0.47 | 0.68 | 0.31 | 0.43 | 0.40 | 0.63 | 0.33 |
| 0.60 | 0.47 | 0.68 | 0.31 | 0.43 | 0.40 | 0.63 | 0.33 |
| 0.21 | 0.37 | 0.12 | 0.07 | 0.17 | 0.16 | Spot vanished | 0.05 |
| 0.21 | 0.37 | 0.12 | 0.07 | 0.17 | 0.16 | Spot vanished | 0.05 |
| 1.00 | 0.00 | 0.04 | 0.83 | 1.00 | 1.00 | 1.00 | 0.82 |
| 1.00 | 0.00 | 1.00 | 0.56 | 0.77 | 0.74 | 0.80 | 0.61 |

## TABLE 11

### $R_F$ VALUES OF SOME FATTY ACIDS

(M. ROMANUK, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 2064)

Solvent: 2-Ethylhexanol-1 (prepared by saponification of commercial dioctylphthalate b.p. 184–185°) saturated with 5 *N* ammonia (upper phase).

Paper: Whatman No. 1 (descending, 18 h, ~ 30 cm run, dried 5-7 h in a stream of air at room temperature).

Temperature: 18–20°.

Detection: Aqueous solution of chlorophenol red (spray).

| *Acid* | $R_F$ |
|---|---|
| Capronic acid | 0.11 |
| Oenanthic acid | 0.30 |
| Caprylic acid | 0.55 |
| Pelargonic acid | 0.74 |
| Caprinic acid | 0.83 |
| Undecylic acid | 0.89 |

## TABLE 12

### $R_F$ VALUES OF FAT-SOLUBLE DYES

(M. R. VERMA AND RAMJI DAS, *J. Sci. Ind. Research* (*India*), 17B (1958) 304)

Paper: Whatman No. 1 dipped into a 5 % solution of Dow Corning silicone fluid 1107 in petroleum ether and dried at 110° for 2 hours.
Technique: ascending. Temperature: 35°.
Solvents: A. Ethyl alcohol (60), water (40), saturated with toluene. B. Ethyl alcohol (60), water (40), methyl isobutyl ketone (3.5). C. Ethyl alcohol (60), water (40), tetrachloroethane (15). D. Ethyl alcohol (56), water (44), chloroform (15). E. Ethyl alcohol (60), water (40), saturated with dichlorobenzene. F. Ethyl alcohol (58), water (42), saturated with $\alpha$-bromonaphthalene. G. Ethyl alcohol (60), water (40), saturated with benzyl benzoate.

| *Dye* | $R_F$ *values* | | | | | | |
|---|---|---|---|---|---|---|---|
| | *A* | *B* | *C* | *D* | *E* | *F* | *G* |
| *Yellow* | | | | | | | |
| Fat Yellow S.G. | 0.00 | 0.00 | 0.16 | 0.00 | 0.00 | 0.00 | 0.00 |
| Oil Yellow Saco | 0.53 | 0.54 | 0.43 | 0.29 | 0.29 | 0.49 | Spot vanishes |
| F.D. & C. Yellow No. 4 | 0.56 | 0.57 | 0.52 | 0.38 | 0.32 | 0.46 | 0.74 |
| F.D. & C. Yellow No. 3 | 0.67 | 0.68 | 0.59 | 0.44 | 0.41 | 0.58 | 0.64 |
| Spirit Yellow (C.I. 17) | 0.80 | 0.79 | 0.68 | 0.72 | 0.75 | 0.77 | 0.91 |
| Organol Yellow A.N.P. | 0.87 | 1.00 | 0.75 | 0.90 | 0.85 | 0.87 | 0.94 |
| Ceres Orange G (C.I. 23) | 1.00 | 0.91 | 0.94 | 0.94 | 0.83 | 0.92 | 0.90 |
| | Tailing | 0.51 | 0.27 | 0.27 | 0.16 | Tailing | 0.38 |
| Waxoline Yellow O.S. | 0.90 | 1.00 | Tailing | 1.00 | 0.94 | 0.84 | 1.00 |
| Oil Yellow B (C.I. 15) | 0.90 | 1.00 | 0.81 | 0.90 | 0.94 | 0.92 | 1.00 |
| Martius Yellow | 0.90 | 1.00 | 1.00 | 0.90 | 0.95 | 1.00 | 1.00 |
| Chrysoidine (C.I. 20) | 0.86 | 1.00 | 0.95 | 0.90 | 0.94 | 1.00 | 1.00 |
| *Orange* | | | | | | | |
| F.D. & C. Orange No. 2 | 0.37 | 0.52 | 0.25 | 0.25 | 0.19 | 0.31 | 0.38 |
| Fat Orange 4A (C.I. 24) | 0.44 | 0.56 | 0.29 | 0.29 | 0.23 | 0.40 | 0.50 |
| *Red* | | | | | | | |
| F.D. & C. Red No. 32 | 0.31 | 0.43 | 0.18 | 0.18 | 0.14 | 0.22 | 0.27 |
| Fat Red 7B | 0.12 | 0.21 | 0.28 | 0.07 | 0.03 | 0.05 | 0.05 |
| Red Powder H 4887 | Tailing | Tailing | 0.28 | Tailing | 0.08 | 0.05 | 0.12 |
| Organol Red B.S. (C.I. 258) | 0.15 | 0.55 | 0.23 | Spot vanishes | 0.33 | 0.41 | 0.47 |
| | | 0.25 | | 0.09 | 0.04 | 0.05 | 0.05 |
| Fat Red B | 0.40 | 0.55 | 0.26 | 0.26 | 0.13 | 0.35 | 0.37 |
| Ceres Red G (C.I. 113) | 0.60 | 0.62 | 0.49 | 0.41 | 0.38 | 0.51 | 0.61 |
| Fast Pink B | 1.00 | 1.00 | 0.32 | 1.00 | 1.00 | 1.00 | 1.00 |
| *Brown* | | | | | | | |
| Fat Brown 3R | 0.86 | 0.78 | 0.75 | 0.67 | 0.65 | 0.71 | 0.79 |
| | 0.00 | 0.00 | 0.45 | 0.31 | Spot vanishes | 0.33 | Spot vanishes |
| Oil Brown 58217 | 0.85 | 0.81 | 0.75 | 0.80 | | 0.74 | 0.87 |
| | 0.39 | 0.55 | 0.47 | 0.25 | Tailing | Spot vanishes | 0.36 |
| | 0.00 | 0.00 | 0.00 | 0.00 | | | 0.00 |
| Oil Brown (C.I. 81) | 0.30 | 0.36 | 0.46 | 0.17 | Spot vanishes | 0.18 | 0.30 |
| | 0.00 | 0.00 | 0.00 | 0.00 | | 0.00 | 0.00 |

## TABLE 13

### $R_F$ VALUES OF PHENOXYACETIC ACIDS

(L. S. BARK AND R. J. T. GRAHAM, *Analyst*, 84 (1959) 454)

Paper: Whatman No. 1.
Solvents: 1. *n*-Butanol–water.
2. *n*-Butanol–1.5 *N* ammonia.
3. *n*-Butanol–3.0 *N* ammonia.

| Acid | $R_F$ | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Phenoxyacetic | 0.22 | 0.23 | 0.33 |
| *o*-Chlorophenoxyacetic | 0.295 | 0.30 | 0.41 |
| *p*-Chlorophenoxyacetic | 0.29 | 0.32 | 0.42 |
| 2,4-Dichlorophenoxyacetic | 0.37 | 0.41 | 0.495 |
| 2,4,6-Trichlorophenoxyacetic | 0.43 | 0.50 | 0.55 |
| *p*-Methylphenoxyacetic | 0.27 | 0.315 | 0.36 |
| 2,4-Dimethylphenoxyacetic | 0.365 | 0.42 | 0.45 |
| 2,3,5-Trimethylphenoxyacetic | 0.405 | 0.47 | 0.50 |

## TABLE 14

### $R_F$ VALUES OF SOME TOSYL DERIVATIVES OF AMINO ACIDS AND RELATED COMPOUNDS

(M. ZAORAL AND J. RUDINGER, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 1993)

Solvent: $S_1$ = *n*-Butanol–acetic acid–water (4:1:5, upper phase).
$S_2$ = Phenol–water–ammonia.
$S_3$ = Methyl ethyl ketone–acetone–water–formic acid (3:1:1:0.1).
Paper: Whatman No. 4.
Detection: Ninhydrin.

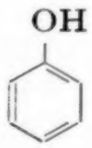
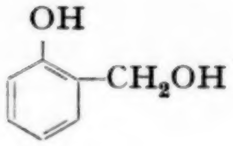
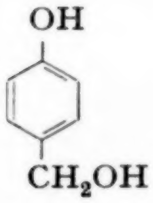
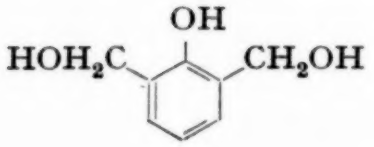
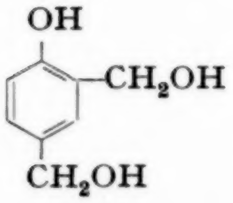
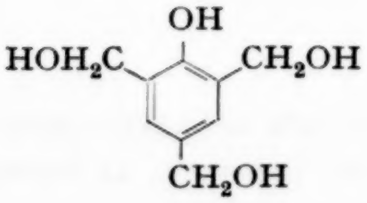
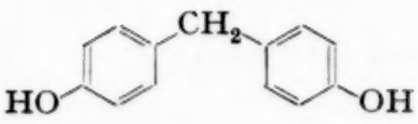
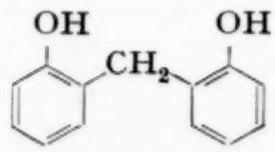
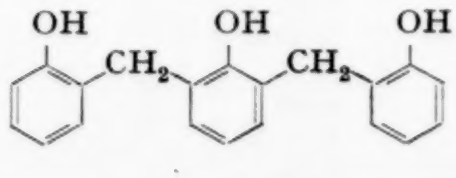
| Compound | $R_F$ | | |
|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ |
| $N^{\alpha}$-Tosyl-L-$\alpha,\gamma$-diaminobutyric acid | 0.53 | | |
| Methyl $N^{\alpha}$-tosyl-L-$\alpha,\gamma$-diaminobutyrate hydrochloride | 0.69 | | |
| L-$\alpha,\gamma$-Diaminobutyric acid | | 0.75 | 0.55 |
| $N^{\alpha}$-Tosyl-L-ornithine | 0.46 | | |
| $N^{\alpha}$-Tosyl-L-ornithine methyl ester hydrochloride | 0.57 | | |
| $N^{\alpha}$-Phthaloyl-DL-ornithine methyl ester hydrochloride | 0.66 | | |

## TABLE 15

### $R_F$ VALUES OF REACTION PRODUCTS OF PHENOL WITH FORMALDEHYDE

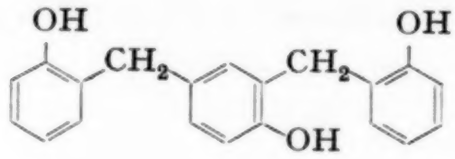
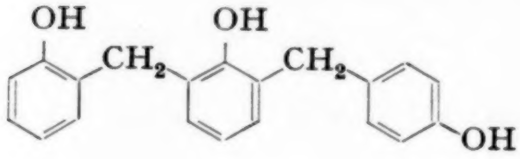
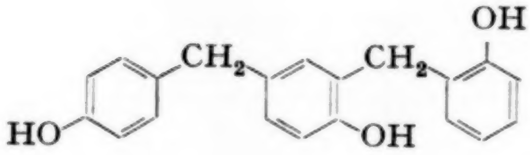
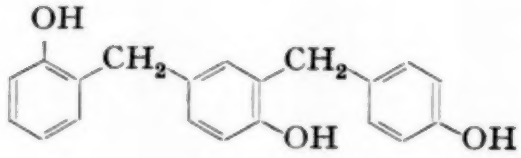
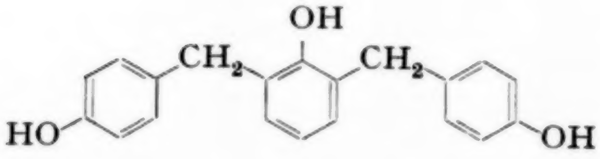
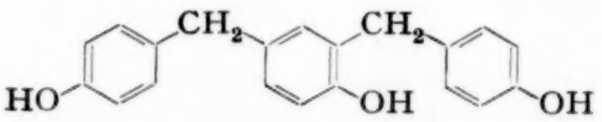
(H. G. PEER, *Rec. trav. chim.*, 78 (1959) 631)

Paper: Whatman No. 1.
Solvents: 1. Butanol–$NH_3$ (4:1).
2. Benzene–acetic acid–water (10:4:1).

| *Compound* | $R_F$ | |
|---|---|---|
| | *1* | *2* |
|  | appr. 0.90 | 0.90 |
|  | 0.81 | 0.36 |
|  | 0.75 | 0.12 |
|  | 0.65 | 0.15 |
|  | 0.58 | 0.05 |
|  | 0.33 | 0.02 |
|  | appr. 0.90 | 0.54 |
|  | appr. 0.90 | 0.98 |
|  | appr. 0.90 | 0.96 |

*(continued on p. D15)*

TABLE 15 (*continued*)

| *Compound* | $R_F$ | |
|---|---|---|
| | *1* | *2* |
|  | appr. 0.90 | 0.80 |
|  | appr. 0.90 | 0.94 |
|  | appr. 0.90 | 0.65 |
|  | appr. 0.90 | 0.33 |
|  | appr. 0.90 | 0.49 |
|  | appr. 0.90 | 0.19 |

## TABLE 16

### $R_F$ VALUES OF FERROCENE AND ITS DERIVATIVES

(A. N. DE BELDER, E. J. BOURNE AND J. B. PRIDHAM, *Chem. & Ind.* (*London*), (1959) 996)

Paper: Whatman No. 3 dipped twice in a solution of dimethyl sulphoxide in benzene (20% v/v) and heated in an oven at 80° for approx. 30 sec.
Solvent: Light petroleum (b.p. 60–80°) saturated with dimethyl sulphoxide.

| *Compound* | $R_F$ *value* |
|---|---|
| Ferrocene | 0.98 |
| Acetylferrocene | 0.62 |
| Ferrocenealdehyde | 0.59 |
| Hydroxymethylferrocene | 0.19 |
| Ferrocenecarboxylic acid | 0.05 |

## TABLE 17

### HIGH VOLTAGE PAPER ELECTROPHORESIS OF ORGANIC ACIDS

(D. GROSS, *Chem. & Ind.* (*London*), (1959) 1219)

Electrolytes: (1) 0.75 $M$ formic acid solution of pH 2.0.
(2) 0.5 $M$ acetic acid solution adjusted with pyridine to pH 4.0.
(3) Approx. 0.1 $M$ ammonium carbonate solution (7.9 g/l) of pH 8.9.

Conditions: (a) For electrolyte (1) 100 V/cm, 6.5 mA/cm, 25 min, 15.5°.
(b) For electrolyte (2) 100 V/cm, 9.6 mA/cm, 20 min, 16°.
(c) For electrolyte (3) 80 V/cm, 8 mA/cm, 25 min, 8°.

Migration rates are relative to the rate of the chloride ion: $M_{Cl}$.

| *Acid* | $M_{Cl}$ | | |
|---|---|---|---|
| | *pH 2.0* | *pH 4.0* | *pH 8.9* |
| $Cl^-$ | 1.00 | 1.00 | 1.00 |
| Trichloroacetic | 0.43 | 0.39 | 0.37 |
| Dichloroacetic | 0.41 | 0.45 | 0.42 |
| Oxalic | 0.41 | 0.48 | 0.70 |
| Meconic | 0.40 | 0.42 | 0.27 |
| Maleic | 0.28 | 0.45 | 0.59 |
| 3-Phosphoroglyceric | 0.28 | 0.43 | 0.48 |
| *cis*-Aconitic | 0.23 | 0.38 | 0.63 |
| Oxalosuccinic | 0.17 | 0.40 | 0.57 |
| α-Ketoglutaric | 0.17 | 0.40 | 0.57 |
| Oxalacetic | 0.16 | 0.34 | 0.48 |
| Pyruvic | 0.16 | 0.48 | 0.50 |
| Dihydroxytartaric | 0.15 | 0.45 | 0.63 |
| Citraconic | 0.13 | 0.35 | 0.53 |
| α-Ketobutyric | 0.13 | 0.28 | 0.42 |
| Malonic | 0.10 | 0.44 | 0.63 |
| Monochloroacetic | 0.08 | 0.46 | 0.45 |
| Fumaric | 0.06 | 0.40 | 0.61 |
| Tartronic | 0.06 | 0.40 | 0.57 |
| *trans*-Aconitic | 0.05 | 0.38 | 0.63 |
| Tartaric | 0.05 | 0.40 | 0.59 |
| 2-Ketogluconic | 0.05 | 0.28 | 0.25 |
| Glyoxylic | 0.04 | 0.39 | 0.57 |
| Citric | 0.04 | 0.30 | 0.45 |
| Mesaconic | 0.04 | 0.34 | 0.57 |
| Galacturonic | 0.03 | 0.22 | 0.22 |
| Malic | 0.03 | 0.32 | 0.57 |
| Dihydroxymaleic | 0.03 | 0.38 | 0.66 |
| β-Hydroxybutyric | 0.02 | 0.12 | 0.35 |
| Tricarballylic | 0.02 | 0.23 | 0.59 |
| Glycollic | 0.02 | 0.31 | 0.48 |
| Ascorbic | 0.02 | 0.12 | 0.25 |
| Lactic | 0.02 | 0.25 | 0.42 |
| Adipic | 0.02 | 0.10 | 0.48 |
| Itaconic | 0.02 | 0.23 | 0.57 |
| Glutaric | 0.02 | 0.13 | 0.53 |
| Gluconic | 0.02 | 0.20 | 0.25 |
| Laevulinic | 0.02 | 0.09 | 0.35 |
| Acrylic | 0.01 | 0.14 | 0.45 |
| Succinic | 0.01 | 0.18 | 0.57 |
| Sorbic | 0.01 | 0.05 | 0.32 |
| Pimelic | 0.01 | 0.09 | 0.45 |
| Suberic | 0.01 | 0.08 | 0.45 |
| Sebacic | 0.01 | 0.05 | 0.41 |

*(continued on p. D17)*

TABLE 17 (*continued*)

| *Acid* | $M_{Cl}$ | | |
|---|---|---|---|
| | *pH 2.0* | *pH 4.0* | *pH 8.9* |
| $Cl^-$ | 1.00 | 1.00 | 1.00 |
| Sulphosalicylic | 0.35 | 0.41 | 0.53 |
| Picric | 0.26 | 0.23 | 0.22 |
| 3-Nitrosalicylic | 0.19 | 0.21 | 0.24 |
| 5-Nitrosalicylic | 0.13 | 0.22 | 0.22 |
| Phthalic | 0.05 | 0.27 | 0.45 |
| Salicylic | 0.04 | 0.24 | 0.32 |
| Sulphanilic | 0.03 | 0.30 | 0.37 |
| Pyrrolidone-carboxylic | 0.03 | 0.31 | 0.35 |
| Syringic | 0.01 | 0.03 | 0.22 |
| Benzoic | 0.01 | 0.14 | 0.37 |
| Tannic | 0.01 | 0.03 | 0.29 |

## TABLE 18

### PAPER ELECTROPHORESIS OF CARBOHYDRATES IN MOLYBDATE SOLUTIONS

(E. J. BOURNE, D. H. HUTSON AND H. WEIGEL, *Chem. & Ind. (London)*, (1959) 1047)

Paper: Whatman No. 3 MM.
Conditions: 15 V/cm.
Electrolyte: 0.1 *M* sodium molybdate adjusted to pH 5 with sulphuric acid.
$M_S$ value: D-glucitol was used as standard and glycerol as non-migrating marker to correct for electro-osmosis.

| *Compound* | $M_S$ *value* | *Compound* | $M_S$ *value* |
|---|---|---|---|
| D-Glucitol | 1.0 | Turanose | 0.14 |
| D-Mannitol | 1.0 | Leucrose | 0.43 |
| Dulcitol | 1.0 | Sophorose | $< 0.1$ |
| Ribitol | 1.1 | Nigerose | $< 0.1$ |
| D-Arabitol | 1.1 | Laminaribiose | $< 0.1$ |
| Xylitol | 1.1 | Maltose | $< 0.1$ |
| D-Altrose | $< 0.1$ | Cellobiose | $< 0.1$ |
| D-Glucose | $< 0.1$ | Lactose | $< 0.1$ |
| D-Mannose | $0 \rightarrow 0.9$ | Isomaltose | $< 0.1$ |
| D-Gulose | 1.1 | Gentiobiose | $< 0.1$ |
| D-Galactose | $< 0.1$ | Melibiose | $< 0.1$ |
| D-Talose | 0.7 | Sophoritol ($\beta$-1:2)* | 0.9 |
| D-Ribose | 0.4 | Nigeritol ($\alpha$-1:3)* | 0 |
| D-Arabinose | $< 0.1$ | Laminaribiitol ($\beta$-1:3)* | 0 |
| D-Xylose | $< 0.1$ | Maltitol ($\alpha$-1:4)* | 0.4 |
| D-Lyxose | 1.1 | Cellobiitol ($\beta$-1:4)* | 0.4 |
| D-Erythrose | 0.9 | Lactitol ($\beta$-1:4)* | 0.4 |
| D-Threose | 0.6 | Isomaltitol ($\alpha$-1:6)* | 0.8 |
| D-Fructose | 0.5 | Gentiobiitol ($\beta$-1:6)* | 0.8 |
| L-Sorbose | 0.3 | Melibiitol ($\beta$-1:6)* | 0.8 |

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* Glycosidic link in parentheses.

## TABLE 19

### PAPER ELECTROPHORESIS OF CARBOHYDRATES

(J. L. FRAHN AND J. A. MILLS, *Australian J. Chem.*, 12 (1959) 65)

Paper: Whatman No. 4.
$M_G$ value = movement relative to glucose.
$M_R$ value = movement relative to ribose.
Ninety-minute runs at 20–25 V/cm, with room temperature 20–25° and cooling water 18–20°.

| *Compounds tested* | *Relative rates of migration in principal electrolytes* | | | |
|---|---|---|---|---|
| | *Borax 0.05 M* ($M_G \times 10^2$) | *Sodium arsenite pH 9.6** ($M_R \times 10^2$) | *Sodium hydroxide 0.1 N* ($M_R \times 10^2$) | *Basic lead acetate*** ($M_R \times 10^2$) |
| *Reducing sugars* | | | | |
| L-Arabinose | 91 | 30 | 79 | 7 |
| D-Lyxose | 71 | 42 | 97 | 30 |
| D-Ribose | 75 | 100 | 100 | 100 |
| D-Xylose | 101 | 17 | 93 | 8 |
| L-Fucose | 83*E**** | 22 | 60 | 6 |
| L-Rhamnose | 49 | 32 | 88 | 28 |
| D-Allose | 83 | 75 | 68 | 33 |
| D-Altrose | 97 | 77 | 96 | 10 |
| D-Galactose | 93 | 28 | 65 | 10 |
| D-Glucose | 100 | 16 | 80 | 6 |
| D-Gulose | 82 | 53 | 70 | 31 |
| L-Idose | 102 | 115 | 96 | 42 |
| D-Mannose | 69 | 35 | 84 | 41 |
| D-Talose | 87 | 119 | 103 | 110 |
| D-Erythropentulose | 90 | 209 | — | 73 |
| D-Threopentulose | 75 | 194 | — | 41 |
| D-Fructose | 89 | 75 | 89 | 22 |
| D-Psicose | 76 | 188 | 125 | 91*S**** |
| L-Sorbose | 97 | 73 | 88 | 16 |
| D-Tagatose | 95 | 103 | 82 | 65 |
| D-Glycero-L-glucoheptose | 104 | 23 | 72 | 7 |
| 3-O-Methyl-D-glucose | 76 | 13 | 77 | 0 |
| 2,3,6-Tri-O-methyl-D-glucose | 0† | 0† | 71 | 0† |
| 2,4,6-Tri-O-methyl-D-glucose | 0† | 0† | 66 | 0† |
| 3,5,6-Tri-O-methyl-D-glucose | 65 | 134 | — | 8 |
| Tetra-O-methyl-D-glucopyranose | 0† | 0† | 74 | 0† |
| Cellobiose | 22 | 15 | 68 | 10 |
| Lactose | 37 | 24 | 61 | 13 |
| Maltose | 30 | 15 | 68 | 7 |
| Melibiose | 77 | 32 | 62 | 10 |
| Turanose | 64 | 30 | 79 | 7 |
| *Glycosides* | | | | |
| Sucrose | 16 | 14 | 29 | 4 |
| Raffinose | 26 | 25 | 30 | 7 |
| Methyl α-D-altropyranoside | 58 | — | 7 | 1 |
| Methyl α-D-galactopyranoside | 35 | 19 | 6 | 5 |
| Methyl β-D-galactopyranoside | 34 | 19 | 7 | 5 |
| Methyl α-D-glucopyranoside | 10 | 9 | 9 | 1 |
| Methyl β-D-glucopyranoside | 15 | 6 | 9 | 3 |
| Methyl α-D-mannopyranoside | 39 | 17 | 9 | 0 |
| Phenyl β-D-glucopyranoside | 15 | 12 | 9 | 4 |
| *Sugar alcohols* | | | | |
| Glycerol | 49 | 24 | 0 | 3 |
| Erythritol | 75 | 53 | 3 | 3 |
| L-Threitol | 75 | 96 | 3 | 11 |
| D-Arabitol | 87 | 124 | 7 | 14 |
| Ribitol | 85 | 76 | 10 | 4 |
| Xylitol | 79 | 155 | 3 | 25 |

*(continued on p. D19)*

TABLE 19 *(continued)*

| *Compounds tested* | *Relative rates of migration in principal electrolytes* | | | |
|---|---|---|---|---|
| | *Borax 0.05 M* ($M_G \times 10^2$) | *Sodium arsenite pH 9.6** ($M_R \times 10^2$) | *Sodium hydroxide 0.1 N* ($M_R \times 10^2$) | *Basic lead acetate*** ($M_R \times 10^2$) |
| Allitol | 90 | 92 | 23 | 9 |
| Dulcitol | 97 | 145 | 8 | 32 |
| D-Glucitol | 83 | 161 | 11 | 47 |
| L-Iditol | 81 | 173 | 7 | 57 |
| D-Mannitol | 91 | 130 | 12 | 23 |
| D-Talitol | 89 | 138 | 16 | 17 |
| D-Glycero-D-altroheptitol | 92 | 144 | 44 | 27 |
| D-Glycero-D-galactoheptitol | 98 | 140 | 11 | 51*E**** |
| D-Glycero-D-glucoheptitol | 88 | 171 | 30 | 53*E* |
| D-Glycero-L-glucoheptitol | 95 | 176 | 17 | 59*E* |
| D-Glycero-D-idoheptitol | 85 | 168 | 20 | 71*E* |
| D-Glycero-D-taloheptitol | 93 | 140 | 24 | 34 |
| *meso*-Glycero-alloheptitol | 95 | 100 | 54 | 11 |
| *meso*-Glycero-guloheptitol | 85 | 160 | 27 | 72*E* |
| *meso*-Glycero-idoheptitol | 78 | 182 | 17 | 79*E* |
| *Other glycols* | | | | |
| Ethylene glycol | 11 | 3 | — | 0 |
| Propane-1,2-diol | 16 | 5 | 0 | 0 |
| Propane-1,3-diol | 5 | 0 | — | — |
| Butane-1,3-diol | 10 | 0 | — | — |
| Erythrobutane-2,3-diol | 13 | 6 | 0 | 0 |
| L-Threobutane-2,3-diol | 51 | 33 | 0† | 0 |
| Butane-1,4-diol | 0 | 0 | — | — |
| Erythropentane-2,4-diol | 18 | 0 | 0 | 0 |
| Threopentane-2,4-diol | 0 | 0 | — | — |
| Pentane-1,5-diol | 0 | 0 | — | — |
| 2-Methylpentane-1,3-diol | 8; 24 | 0 | — | — |
| Pentaerythritol | 85 | 21 | 12 | 0 |
| Poly(vinyl alcohol) | 52*E* | 68*E* | 11*E* | 0 |
| *cis*-Cyclohexane-1,2-diol | 6 | 9 | 0 | 0 |
| *trans*-Cyclohexane-1,2-diol | 0 | 2 | — | 0 |
| *Cyclitols* | | | | |
| *allo*-Inositol | 85 | 50 | 9 | 62 |
| *cis*-Inositol | 79 | 40 | 9 | 116 |
| *epi*-Inositol | 76 | 29 | 5 | 74*S**** |
| (—)-Inositol | 59 | 23 | 3 | 20 |
| *muco*-Inositol | 97 | 36 | 15 | 41 |
| *myo*-Inositol | 49*E* | 16 | 2 | 75*E* |
| *neo*-Inositol | 59 | 43 | 1 | 64 |
| *scyllo*-Inositol | 2 | 7 | 3 | Adsorbed |
| 3-O-Methyl-(+)-inositol | 59 | 35 | — | 2 |
| 2-O-Methyl-(—)-inositol | 24 | 27 | — | 14 |
| 1-O-Methyl-*myo*-inositol | 11 | 20 | — | 26 |
| 5-O-Methyl-*myo*-inositol | 15 | 25 | — | 22 |
| *Miscellaneous carbohydrates* | | | | |
| Levoglucosan | 0 | 0 | 5 | 8 |
| 1,2-O-Isopropylidene-D-glucose | 71 | 25 | 31 | 0 |
| 1,2-O-Cyclohexylidene-D-mannitol | 63 | 81 | — | 3 |
| 3,4-O-Isopropylidene-D-mannitol | 61 | 44 | — | 0 |
| 3,4-O-Methylene-D-mannitol | 66 | 47 | — | 2 |
| 2,5-O-Methylene-D-mannitol | 62 | 28 | — | 0 |
| 2,4-O-Methylene-D-glucitol | 58 | 33 | — | 0 |
| 2-Amino-2-deoxy-D-glucitol | 47 | 51 | 8 | 116 |

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* 19.8 g $As_2O_3$ per l and NaOH added.

** 58 g per l.

*** *E*, markedly elongated spot; *S*, extensive streaking.

† Non-migrating by definition.

## TABLE 20

## $R_G$ VALUES OF MONO- AND DIGLYCOSYL UREAS

(M. H. Benn and A. S. Jones, *Chem. & Ind.* (*London*), (1959) 997)

Paper: Whatman No. 1.
Solvent: *n*-Butanol–acetic acid–water (4:1:5).

| *Glycosyl ureas* | $R_G$ | *Diglycosyl ureas* | $R_G$ |
|---|---|---|---|
| Glucosyl | 0.63 | Diglucosyl | 0.14 |
| Mannosyl | 0.68 | Dimannosyl | 0.15 |
| Galactosyl | 0.51 | Digalactosyl | 0.09 |
| Rhamnosyl | 1.35 | Dirhamnosyl | 0.52 |
| Ribopyranosyl | 0.92 | Diribosyl | 0.25, 0.34, 0.43 |
| Ribofuranosyl | 1.23 | Diarabinosyl | 0.19, 0.25, 0.33 |
| Arabinosyl | 0.70 | Dixylosyl | 0.28, 0.45 |
| Xylosyl | 0.85 | | |

## TABLE 21

## $R_F$ VALUES OF HYDROPEROXIDIC DERIVATIVES OF THYMINE

(B. Ekert and R. Monier, *Nature*, 184 (1959) B.A. 58)

Paper: Whatman No. 1.
Solvent: *n*-Propanol–1 *N* HCl (85:15).
Temperature: 2°.

| *Thymine derivative* | $R_F$ |
|---|---|
| *cis*-4-Hydroxy-5-hydroperoxythymine | 0.51 |
| *trans*-4-Hydroxy-5-hydroperoxythymine | 0.33 |
| *cis*-4-Hydroperoxy-5-hydroxythymine | 0.62 |
| *trans*-4-Hydroperoxy-5-hydroxythymine | 0.43 |
| *trans*-4-Hydroperoxy-5-bromothymine | 0.83 |
| Hydrogen peroxide | 0.68 |
| Thymine | 0.55 |

## TABLE 22

## $R_F$ VALUES OF HYPONITRITE, NITRITE, NITRATE AND HYDROXYLAMINE

(H. M. Stevens, *Anal. Chim. Acta*, 21 (1959) 456)

Paper: Whatman No. 1.
Solvent: ethanol 70 ml, water 30 ml, sodium hydroxide 2 g.

| | *$R_F$ values (approximate)* |
|---|---|
| Hyponitrite ($N_2O_2^{--}$) | 0.1 |
| $NO_2^-$ | 0.5–0.6 |
| $NO_3^-$ | |
| $NH_2OH$ | |

## TABLE 23

### $R_F$ VALUES OF SOME NAPHTHALENE DERIVATIVES

(J. LATINÁK, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 2939)

Solvents: $S_1$ = Formamide/benzene–ethyl acetate (8:2).
$S_2$ = Formamide/chloroform–ethyl acetate (8:2).
$S_3$ = Formamide/chloroform.
$S_4$ = Formamide/carbon tetrachloride.
$S_5$ = Formamide/cyclohexane.
$S_6$ = Dimethylformamide/cyclohexane.

Paper: Whatman No. 4 (descending 25–30 cm); impregnated by dipping in 10–30% methanolic solution of formamide (or of dimethylformamide).

Detection: diazotised *p*-nitraniline (spray) or ferric chloride–potassium ferricyanide (spray or dip) and U.V.

| *Compound* | $R_F$ | | | | | |
|---|---|---|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ | $S_4$ | $S_5$ | $S_6$ |
| 1-Naphthol | 0.90 | 0.89 | 0.66 | 0.31 | 0.10 | 0.07 |
| 2-Naphthol | 0.87 | 0.87 | 0.62 | 0.21 | 0.06 | 0.04 |
| 1,1′-Dinaphthol | 0.95 | 0.95 | | 0.08 | 0.00 | |
| Unknown substance in 2-naphthol | 0.96 | 0.96 | | 0.59 | 0.21 | |
| 1-Iodo-2-naphthol | | | | 0.83 | 0.63 | |
| 1,3-Dihydroxynaphthalene | 0.30 | 0.21 | | 0.00 | 0.00 | |
| 1,5-Dihydroxynaphthalene | 0.32 | 0.23 | 0.03 | 0.01 | 0.00 | 0.00 |
| 1,6-Dihydroxynaphthalene | 0.27 | 0.22 | | | | |
| 1,7-Dihydroxynaphthalene | 0.27 | 0.22 | | | | |
| 1,8-Dihydroxynaphthalene | 0.80 | 0.81 | 0.48 | 0.02 | 0.00 | 0.02 |
| 2,3-Dihydroxynaphthalene | 0.40 | 0.37 | | | | |
| 2,6-Dihydroxynaphthalene | 0.18 | 0.18 | | | | |
| 2,7-Dihydroxynaphthalene | 0.20 | 0.19 | | | | |
| 1-Naphthylamine | 0.92 | 0.93 | 0.93 | 0.73 | 0.45 | 0.08 |
| 2-Naphthylamine | 0.89 | 0.92 | 0.92 | 0.68 | 0.38 | 0.05 |
| 1,5-Diaminonaphthalene | 0.46 | 0.51 | 0.40 | 0.05 | 0.02 | 0.00 |
| 1,8-Diaminonaphthalene | 0.87 | 0.88 | 0.85 | 0.58 | 0.23 | |
| 1,6-Aminonaphthol | 0.32 | 0.31 | 0.05 | 0.02 | 0.00 | 0.00 |
| 1,7-Aminonaphthol | 0.33 | 0.33 | | | | |
| 1-N-Phenylnaphthylamine | 0.96 | 0.96 | 0.95 | 0.94 | 0.94 | 0.52 |
| 2-N-Phenylnaphthylamine | 0.95 | 0.95 | 0.94 | 0.93 | 0.93 | 0.39 |

## TABLE 24

### $R_F$ VALUES OF TWO N-ALKYL-SULPHONAMIDES

(J. PETRÁNEK AND M. VECERA, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 2191)

Solvents: $S_1$ = Benzene–cyclohexane (3:2)(formamide-impregnated paper).
$S_2$ = 75% ethanol (petroleum-impregnated paper).

Paper: Whatman No. 4.

Detection: Ehrlich reagent after reduction of the nitro group with zinc chloride.

| *Compound* | $R_F$ | |
|---|---|---|
| | $S_1$ | $S_2$ |
| N-Allyl-N-allylthio-*p*-nitrobenzenesulphonamide | 1.00 | 0.51 |
| N-Allyl-N-benzylthio-*p*-nitrobenzenesulphonamide | — | 0.42 |

## TABLE 25

### $R_F$ VALUES OF BASIC DI- AND TRIPHENYL-METHANE DYES AND RELATED SUBSTANCES

(J. GASPARIČ AND M. MATRKA, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 1943)

Solvents: $S_1$ = Ethanol–ammonia–water (2:2:1); paper impregnated with 2% lauryl alcohol.
$S_2$ = Ethanol–ammonia–water (2:2:1); paper impregnated with 5% lauryl alcohol.
$S_3$ = Ethanol–ammonia (1:1); paper impregnated with 5% lauryl alcohol.
$S_4$ = Ethanol–5% KCl (1:1); paper impregnated with 5% lauryl alcohol.
(Mobile phases $S_1$–$S_4$ were saturated with lauryl alcohol before use.)
Paper: Whatman No. 3, 13 × 40 cm (descending).
Temperature: Room temperature.
Detection: 1% potassium dichromate–1% oxalic acid mixture (1:1) (spray) for leucobases. Removal of excess ammonia (drying in the presence of hydrochloric acid or acetic acid fumes if necessary) reveals the coloured spots; U.V.

| *Compound* | $R_F$ | | | |
|---|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ | $S_4$ |
| Methane base | 0.05 | 0.02 | 0.17 | 0.07 |
| Michler's hydrol | 0.11 | 0.06 | 0.22 | — |
| Bis-(4,4′-bis-dimethylamino-benzhydryl) ether | 0.54 | 0.36 | 0.54 | — |
| Tetraethyldiaminodiphenyl-carbinol | 0.01 | 0.00 | — | — |
| Michler's ketone | 0.33 | 0.18 | 0.45 | 0.24 |
| Auramine O | 0.25 | 0.14 | 0.38 | 0.84 |
| Auramine G | 0.48 | 0.30 | 0.56 | 0.84 |
| Döbner's Violet | 0.77 | 0.68 | 0.75 | 0.80 |
| Malachite Green (oxalate) | 0.14 | 0.06 | 0.29 | 0.80 |
| Malachite Green (perchlorate) | 0.14 | 0.06 | 0.29 | — |
| Malachite Green (chloride) | 0.14 | 0.06 | 0.29 | — |
| Malachite Green (carbinol base) | 0.14 | 0.06 | 0.29 | — |
| N,N-Dimethyl-N′,N′-diethyl-diaminotriphenyl-carbinol | 0.04 | 0.02 | 0.15 | — |
| Brilliant Green | 0.02 | 0.01 | 0.08 | — |
| Döbner's Violet leucobase | 0.63 | 0.43 | 0.67 | 0.59 |
| Malachite Green leucobase | 0.03 | 0.00 | 0.11 | — |
| Setoglaucine O | 0.08 | 0.04 | 0.09 | — |
| | 0.26 | 0.11 | 0.23 | — |
| | 0.41 | 0.26 | 0.37 | — |
| | 0.61 | — | 0.53 | — |
| Turquoise Blue (extra) B | 0.07 | 0.01 | 0.09 | — |
| | 0.14 | 0.05 | 0.16 | — |
| | 0.32 | 0.14 | 0.28 | — |
| | 0.50 | 0.31 | 0.43 | — |
| | 0.67 | — | 0.58 | — |
| Parafuchsine | 0.77 | 0.73 | 0.71 | 0.80 |
| Methyl Violet | 0.18 | 0.09 | 0.32 | — |
| | 0.38 | 0.19 | 0.47 | — |
| | 0.58 | 0.38 | 0.60 | — |
| | 0.70 | 0.53 | 0.71 | — |
| Crystal Violet | 0.18 | 0.09 | 0.32 | 0.80 |
| Methyl Green | 0.26 | 0.14 | 0.32 | 0.33 |
| Fuchsine | 0.85 | 0.80 | 0.80 | 0.85 |
| Neofuchsine | 0.85 | 0.80 | 0.80 | 0.85 |
| Parafuchsine leucobase | 0.78 | 0.77 | 0.79 | 0.78 |
| Crystal Violet leucobase | 0.04 | 0.02 | 0.15 | 0.06 |
| Victoria Blue B | 0.09 | 0.00 | 0.13 | 0.32 |
| Aniline Blue B | 0.02 | 0.00 | 0.10 | 0.00 |
| Night Blue | 0.02 | 0.00 | 0.02 | 0.07 |

## TABLE 26

### $R_F$ VALUES OF SOME TRIPHENYLMETHANE DYES AND THEIR LEUCOBASES

(J. GASPARIČ AND M. MATRKA, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 1943)

Solvent: Benzene using paper impregnated with formamide.
Paper: Whatman No. 4 impregnated by dipping in a 10% formamide solution (descending).

| *Compound* | $R_F$ |
|---|---|
| Döbner's Violet | 0.00 |
| Döbner's Violet leucobase | 0.90 |
| Parafuchsine | 0.00 |
| Parafuchsine leucobase | 0.40 |
| Malachite Green | 0.00 |
| Malachite Green leucobase | 0.96 |
| Crystal Violet | 0.00 |
| Crystal Violet leucobase | 0.96 |
| Michler's ketone | 0.95 |

## TABLE 27

### INFLUENCE OF PAPER IMPREGNATION ON $R_F$ VALUES OF TRIPHENYLMETHANE DYES

(J. GASPARIČ AND M. MATRKA, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 1943)

Solvent: Ethanol–ammonia–water (2:2:1).
Paper: Whatman No. 4 (descending), impregnated with lauryl alcohol.

| *Dye* | $R_F$ | | |
|---|---|---|---|
| | | *Degree of impregnation* | |
| | *2%* | *5%* | *10%* |
| Malachite Green | 0.14 | 0.06 | 0.03 |
| Crystal Violet | 0.18 | 0.09 | 0.03 |
| | 0.08 | 0.04 | 0.01 |
| Setoglaucine O | 0.26 | 0.11 | 0.05 |
| | 0.41 | 0.26 | 0.13 |

## TABLE 28

### $R_F$ VALUES OF PIPTANTHINE AND DIACETYLPIPTANTHINE

(U. EISNER AND F. ŠORM, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 2348)

Solvent: *n*-amyl alcohol–acetic acid–water (5:1:4).
Paper: Whatman No. 2.
Detection: Dragendorff reagent.

| *Compound* | $R_F$ |
|---|---|
| Piptanthine | 0.20 |
| Diacetylpiptanthine | 0.34 |

## TABLE 29

### $R_F$ VALUES OF A NUMBER OF YOHIMBINE DERIVATIVES

(Z. J. VEJDĚLEK AND K. MACEK, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 2493)*

Solvents: $S_1$ = Chloroform; paper impregnated with stationary phase.
$S_2$ = Chloroform–benzene (1:1); paper impregnated with stationary phase.
Paper: Paper used is impregnated with 50% ethanolic formamide solution (with 5% ammonium formate added).
Detection: U.V. (green fluorescence) and Dragendorff reagent (spray).

| *Compound* | $R_F$ | |
|---|---|---|
| | $S_1$ | $S_2$ |
| Yohimbyl alcohol | 0.06 | 0.01 |
| Yohimbol | 0.15 | 0.06 |
| 16-Methylyohimbol | 0.27 | 0.12 |
| Acetylyohimbyl alcohol | 0.30 | 0.12 |
| Yohimbine | 0.56 | 0.25 |
| Yohimbone | 0.81 | 0.56 |
| 16-Methylyohimbone | 0.86 | 0.67 |
| Diacetylyohimbyl alcohol | 0.90 | 0.75 |
| Trimethoxybenzoate of yohimbol | 0.94 | — |
| Trimethoxybenzoate of 16-methylyohimbol | 0.96 | 0.92 |
| Reserpine** | 0.96 | 0.92 |

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* First appeared in *Chem. listy*, 52 (1958) 2140.
** For comparison.

## TABLE 30

### $R_F$ VALUES OF 3,4-DIMETHYLANILINE AND SOME RELATED COMPOUNDS

(E. BOYLAND AND P. SIMS, *Biochem. J.*, 73 (1959) 377)

Solvents: $S_1$ = *n*-Butanol saturated with aq. 2 *N* $NH_3$ soln.; 15 h.
$S_2$ = *n*-Butanol–*n*-propanol–water (2:1:1, by vol.); 15 h.
$S_3$ = *n*-Butanol–acetic acid–water (2:1:1, by vol.).
Paper: Whatman No. 1.
Reagents: Ehrlich (*p*-dimethylaminobenzaldehyde, 0.5% in ethanol containing 1 ml conc. HCl/100 ml); or solution of *p*-dimethylaminocinnamaldehyde (2 g) in 6 *N* HCl (100 ml) and ethanol (100 ml); or successively with aq. $NaNO_2$ (0.5%), 0.5 *N* HCl and 2-naphthol (0.5%) in ethanol–2 *N* NaOH (1:1, v/v). Others sprayed with 2 *N* HCl and heated to 70° for 15 min before spraying with previous reagents; U.V.

| *Compound* | $R_F$ | | |
|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ |
| 3,4-Dimethylaniline | 0.93 | 0.91 | 0.83 |
| 3,4-Dimethylacetanilide | 0.88 | 0.88 | 0.92 |
| 2-Amino-4,5-dimethylphenol | 0.82 | 0.78 | 0.84 |
| 2-Amino-4,5-dimethylphenyl sulphate | 0.42 | 0.51 | 0.65 |
| 3,4-Dimethylphenyl sulphate | 0.37 | 0.45 | 0.59 |
| 4-Amino-2-methylbenzoic acid | 0.11 | 0.86 | 0.87 |
| 4-Acetamido-2-methylbenzoic acid | 0.22 | 0.88 | 0.90 |
| Conjugate, probably 4-amino-2-methylbenzoyl-glucosiduronate | 0.07 | 0.20 | 0.53 |

## TABLE 31

### $R_F$ VALUES OF 5-HYDROXYTRYPTAMINE AND RELATED COMPOUNDS

(D. KEGLEVIĆ, Z. SUPEK, S. KVEDER, S. ISKRIĆ, S. KEČKEŠ AND A. KISIĆ, *Biochem. J.*, 73 (1959) 53)

Solvents: $S_1$ = *n*-Butanol–acetic acid–water (4:1:5), 19 h.
$S_2$ = Propan-2-ol–aq. $NH_3$ soln.–water (10:1:1), 22 h.
$S_3$ = Methanol–benzene–*n*-butanol–water (4:2:2:2), 14 h.
$S_4$ = *n*-Butanol–ethanol–water (4:1:1), 20 h.
Paper: Whatman No. 1 (descending).
Detection: Radioautography (Ferrania X-ray film; 5–10 days); Geiger-Müller mica window counter (with automatic recorder) for $^{14}C$; Ehrlich reagent.

| *Compound* | $R_F$ | | | |
|---|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ | $S_4$ |
| 5-Hydroxytryptamine | 0.50 | 0.61 | 0.68 | 0.52 |
| 5-Hydroxyindolylacetic acid | 0.78 | 0.32 | 0.68 | 0.52 |
| 5-Hydroxyindolylaceturic acid | 0.63 | 0.35 | 0.49 | 0.19 |

## TABLE 32

### $R_F$ VALUES OF CERTAIN KETO ACIDS, THEIR THIOSEMICARBAZONES AND SOME URACIL AND THYMINE ANALOGUES

(J. MORÁVEK, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 2571)*

Solvent: *n*-Butanol, saturated with water.
Paper: Whatman No. 4 (descending).
Detection: Geiger-Müller counter VUPEF.20/400A with automatic recorder for $^{14}C$; see also table.

| *Compound* | $R_F$ | *Detection* |
|---|---|---|
| Oxalic acid | 0.0 | Bromocresol Green |
| Glyoxalic acid | 0.73 | Bromocresol Green |
| Glyoxalic acid thiosemicarbazone | 0.59 | Dinitrophenylhydrazine |
| Thiouracil | 0.61 | U.V.; silver chromate** |
| Azauracil | 0.50 | |
| Pyruvic acid | 0.07 | Bromocresol Green |
| Pyruvic acid thiosemicarbazone | 0.73 | Dinitrophenylhydrazine |
| Thioazathymine | 0.75 | U.V.; silver chromate** |
| Azathymine | 0.59 | |

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* First published in *Chem. listy*, 52 (1958) 2147.
** A red-brown complex is formed which is insoluble in dilute nitric acid.

## TABLE 33

### $R_F$ VALUES OF SUCCINIC AND METHYLSUCCINIC ACID

(M. SUCHÝ, V. HEROUT AND F. ŠORM, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 1542)*

Solvent: Butyl acetate saturated with water, containing 1% formic acid.
Paper: Whatman No. 1.

| *Compound* | $R_F$ |
|---|---|
| Succinic acid | 0.19 |
| Methylsuccinic acid | 0.39 |

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* First appeared in *Chem. listy*, 52 (1958) 2110.

## TABLE 34

### $R_F$ VALUES OF URIDINE MONOPHOSPHATE, ADENOSINE MONOPHOSPHATE, URIDINE DIPHOSPHATE AND GUANOSINE-5′-PHOSPHATE

(E. L. DOVEDOVA, *Biokhimiya*, 24 (1959) 414)

Solvents: $S_1$ = Ethanol–pH 3.8 acetate buffer (75:30) (descending).
$S_2$ = *n*-Propanol–$(NH_4)_2SO_4$ (saturated)–pH 6.8 phosphate (0.1 *M*) (2:60:100) (ascending).
$S_3$ = Isobutyric acid–1 *M* $NH_4OH$–0.1 *M* Versene (100:60:1.6) (descending).
Paper: LM-2 (a slow filtering chromatographic paper), washed first in 0.1 *N* HCl then in water (24–40 h run).

| *Solvent system* | $R_F$ | | | | |
|---|---|---|---|---|---|
| | *AMP* | *UMP* | *Guanosine-5′-phosphate* | | *UDP* |
| | | | *(a)** | *(b)** | |
| $S_1$ | 0.71 | 0.83 | 0.68 | 0.55 | 0.58 |
| $S_2$ | 0.25 | 0.64 | 0.54 | 0.41 | 0.71 |
| $S_3$ | 1.0 | 0.49 | 0.60 | 0.41 | 0.24 |

* (a) GMP? (b) GDP?

## TABLE 35

### $R_F$ VALUES OF SOME METHYLATED GUANINES AND THEIR RIBOSIDES

(J. D. SMITH AND D. B. DUNN, *Biochem. J.*, 72 (1959) 294)

Solvents: $S_1$ = Propan-2-ol (680 ml), 11.6 *N* HCl (176 ml), water to 1 litre.
$S_2$ = Propan-2-ol (700 ml), water (300 ml) with $NH_3$ in vapour phase.
$S_3$ = Butan-1-ol (770 ml), water (130 ml), 98% formic acid (100 ml).
$S_4$ = Butan-1-ol (860 ml), water (140 ml) with $NH_3$ in vapour phase.

| *Compound* | $R_F$ | | | |
|---|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ | $S_4$ |
| Guanine | 0.26 | 0.49 | 0.09 | 0.08 |
| 1-Methylguanine | 0.26 | 0.62 | 0.19 | 0.13 |
| 6-Hydroxy-2-methylaminopurine | 0.50 | 0.66 | 0.25 | 0.18 |
| 2-Dimethylamino-6-hydroxypurine | 0.43 | 0.69 | 0.28 | 0.20 |
| 2-Ethylamino-6-hydroxypurine | 0.60 | 0.82 | — | — |
| Guanosine | 0.37 | 0.42 | 0.10 | 0.02 |
| 1-Methylguanosine | 0.45 | 0.64 | 0.16 | 0.11 |
| 6-Hydroxy-2-methylaminopurine riboside | 0.55 | 0.63 | 0.18 | 0.08 |
| 2-Dimethylamino-6-hydroxypurine riboside | 0.49 | 0.66 | 0.23 | 0.10 |
| Uracil | 0.69 | 0.72 | 0.38 | 0.20 |
| Uridine | 0.70 | 0.70 | 0.21 | 0.08 |

## TABLE 36

### $R_F$ VALUES OF GLUTAMIC ACID AND ITS ETHYL ESTERS

(Z. PRAVDA, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 2082)

Solvents: $S_1$ = Phenol–water (3:1).
$S_2$ = *n*-Butanol–acetic acid–water (4:1:1).
Detection: Ninhydrin or Chlorophenol Red.

| *Compound* | $R_F$ | |
|---|---|---|
| | $S_1$ | $S_2$ |
| Glutamic acid | 0.30 | 0.12 |
| $\gamma$-Ethyl L-glutamate | 0.83 | 0.39 |
| $\alpha,\gamma$-Diethyl L-glutamate | 0.92 | 0.75 |

## TABLE 37

### $R_F$ VALUES OF SOME DINITROPHENYL DERIVATIVES

(N. N. MEL'TEVA AND M. S. REZNICHENKO, *Biokhimiya*, 24 (1959) 435)

Solvents: $S_1$ = Aqueous sodium citrate (pH 6).
$S_2$ = Aqueous sodium phosphate (pH 5).
$S_3$ = Aqueous sodium phosphate (pH 6).
$S_4$ = Aqueous sodium phosphate (pH 7).
$S_5$ = Phenol saturated with water.
$S_6$ = Toluene–pyridine–ethylene chlorohydrin–0.8 *N* ammonia (1:1:0.1:0.1, by vol.).

| *Solvent* | *$R_F$ of dinitrophenyl derivatives of* | | | | *$R_F$* | |
|---|---|---|---|---|---|---|
| | *Cystine* | *Glycine* | *Phenylalanine* | *Threonine* | *Dinitrophenol* | *Dinitroaniline* |
| $S_1$ | 0.50 | 0.59 | 0.63 | — | — | 0.28 |
| $S_2$ | 0.62 | 0.67 | 0.72 | — | 0.65 | — |
| $S_3$ | 0.55 | 0.67 | 0.69 | 0.78 | — | 0.33 |
| $S_4$ | 0.56 | 0.65 | 0.65 | — | 0.62 | — |
| $S_5$ | 0.64 | 0.71 | 0.88 | — | 0.53 | 0.9–0.95 |
| $S_6$ | 0.50 | 0.35 | 0.60 | 0.30 | 0.50 | 0.92 |

## TABLE 38

### $R_F$ VALUES OF SOME DINITROPHENYL DERIVATIVES

(N. N. MEL'TEVA AND M. S. REZNICHENKO, *Biokhimiya*, 24 (1959) 435)

Solvents: $S_1$ = Aqueous sodium phosphate (pH 7).
$S_2$ = Phenol saturated with water.
$S_3$ = Toluene–pyridine–ethylene chlorohydrin–0.8 *N* ammonia (1:1:0.1:0.1, by vol.).

| *Solvent* | *$R_F$* | | | | |
|---|---|---|---|---|---|
| | *DNP-oryzenin* | *Mono-DNP-lysine* | *Bis-DNP-histidine* | *Dinitrophenol* | *Dinitroaniline* |
| $S_1$ | 0.70 | 0.71 | 0 | 0.62 | Elongated |
| $S_2$ | 0.81 | 0.83 | 0.94 | 0.54 | 0.90–0.95 |
| $S_3$ | 0.53 | 0.53 | 0.79 | 0.71 | 0.92 |

## TABLE 39

### $R_F$ VALUES OF SOME HYDROXY-AMINO ACIDS

(D. L. SWALLOW AND E. P. ABRAHAM, *Biochem. J.*, 72 (1959) 326)

Solvents: $S_1$ = Butan-1-ol–acetic acid–water (4:1:4, by vol.).
$S_2$ = Butan-1-ol–acetic acid–water (4:1:5, by vol.; organic phase).
$S_3$ = Butan-2-one–propionic acid–water (15:5:6, by vol.).
$S_4$ = Triethylamine–acetone–water (1:16:3, by vol.).
$S_5$ = AnalaR phenol (75 g)–water (21 ml)–aq. $NH_3$ soln. (sp. gr. 0.88; 4.6 ml).
Systems: $S_1$, $S_2$, $S_3$ were freshly prepared. $S_3$ was run 20 h (off paper after 10 h).
Paper: Whatman No. 1 (after $S_4$ and $S_5$ air dried).
Detection: Ninhydrin.
$R_{Val.}$ = $R_F$ relative to $R_F$ of valine.

| *Compound* | $R_F$ | | | | $R_{Val.}$ |
|---|---|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ | $S_4$ | $S_5$ |
| γ-Hydroxy-α-aminobutyric acid | 0.24 | 0.23 | 0.47 | 0.56 | 0.50 |
| γ-Hydroxy-β-aminobutyric acid | 0.31 | 0.28 | 0.41 | 0.60 | 0.71 |
| δ-Hydroxy-α-aminovaleric acid | 0.26 | 0.27 | 0.35 | 0.68 | 0.55 |
| δ-Hydroxy-γ-aminovaleric acid | 0.36 | 0.35 | 0.43 | 0.70 | 0.96 |
| γ-Hydroxy-α-aminobutyric acid lactone* | 0.39 | — | — | — | — |
| γ-Hydroxy-β-aminobutyric acid lactone** | 0.32 | 0.33 | — | — | 1.09 |
| δ-Hydroxy-α-aminovaleric acid lactone | — | — | — | — | — |
| Aspartidiol | 0.36 | 0.35 | 0.57 | — | > 1.5 |
| Leucine and isoleucine | 0.67 | 0.61 | 0.48 | — | 1.32 |
| Phenylalanine | 0.63 | 0.56 | 0.48 | — | 1.32 |

* Grey colour with ninhydrin.
** Yellow colour with ninhydrin.

## TABLE 40

### $R_F$ VALUES OF SOME TOSYL DERIVATIVES OF AMINO ACIDS AND RELATED COMPOUNDS

(K. PODUŠKA AND J. RUDINGER, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 3449)

Solvents: $S_1$ = *n*-Butanol–acetic acid–water (4:1:1).
$S_2$ = Phenol saturated with water–$NH_3$ atmosphere.
Paper: Whatman No. 1 (descending).
Detection: Ninhydrin.

| *Compound* | $R_F$ | |
|---|---|---|
| | $S_1$ | $S_2$ |
| α,γ-Diaminobutyric acid | 0.03 | 0.40 |
| $N^α$-Tosyl-L-α,γ-diaminobutyric acid | 0.20 | 0.88 |
| 1-Tosyl-L-3-aminopyrrolid-2-one hydrobromide | 0.62 | |
| 1-Tosyl-L-3-aminopyrrolid-2-one | | 0.82* |
| $N^γ$-Tosyl-α,γ-diaminobutyric acid | | 0.72* |
| $N^γ$-Tosyl-L-α,γ-diaminobutyric acid | 0.35 | 0.76 |
| $N^γ$-Tosyl-L-α,γ-diaminobutyryl-L-threonine ethyl ester hydrochloride | 0.71 | |

* By circular chromatography.

## TABLE 41

### $R_F$ VALUES OF LOMBRICINE (GUANIDINOETHYL SERYL PHOSPHATE) IN VARIOUS SOLVENT SYSTEMS

(R. PANT, *Biochem. J.*, 73 (1959) 30)

Solvents: Proportions by volume.
$S_1$ = Butan-1-ol–water–acetic acid (73:17:10).
$S_2$ = Pyridine–isoamyl alcohol–water–acetic acid (80:40:40:10).
$S_3$ = Pyridine–isoamyl alcohol–water (80:40:70).
$S_4$ = Pyridine–isoamyl alcohol–water–20% (v/v) aq. $NH_3$, sp. gr. 0.88 (80:40:40:10).
$S_5$ = Phenol saturated with water.
$S_6$ = Propan-1-ol–water–20% (v/v) aq. $NH_3$, sp. gr. 0.88 (73:7:20).
$S_7$ = Methanol–water–acetic acid (80:20:10).
$S_8$ = Phenol (80% w/v)–$NH_3$ atmosphere.
$S_9$ = Aqueous methyl "Cellosolve" (90%) (2-methoxyethanol).
Paper: Whatman No. 1 (25–35 cm, 6–8 h) (ascending).
Temperature: 22°.
Detection: Sakaguchi.

| *Solvent* | $S_1$ | $S_2$ | $S_3$ | $S_4$ | $S_5$ | $S_6$ | $S_7$ | $S_8$ | $S_9$ |
|---|---|---|---|---|---|---|---|---|---|
| $R_F$ | 0.00 | 0.06 | 0.15 | 0.11 | 0.05 | 0.05 | 0.17 | 0.48 | 0.06 |

## TABLE 42

### $R_F$ VALUES OF 2-AMINOETHYL 2-AMINO-2-CARBOXYETHYL HYDROGEN PHOSPHATE (SEP) AND RELATED COMPOUNDS

(H. ROSENBERG AND A. H. ENNOR, *Biochem. J.*, 73 (1959) 521)

Solvents: $S_1$ = Water-saturated phenol (13–14 h).
$S_2$ = Butan-1-ol–acetic acid–water (5:3:2; v/v) (8–9 h).
$S_3$ = Acetone–acetic acid–water (2:2:1; v/v) (3–4 h).
$S_4$ = Diisopropyl ether–acetic acid–ethanol–water (4:4:1:2; v/v) (8–9 h).
$S_5$ = Methyl Cellosolve (2-methoxyethanol)–water (9:1; v/v) (8–9 h).
$S_6$ = Methanol–formic acid–water (8:1:1; v/v) (4 h).
$S_7$ = Ethanol–formic acid–water (7:1:2; v/v) (8–9 h).
$S_8$ = Ethanol–*M* ammonium acetate buffer pH 3.8 (7:3; v/v) (7–8 h).
Paper: Whatman No. 3 washed (ascending).
Detection: 0.2% ninhydrin in acetone; inorganic phosphate detection.

| *Compound* | $R_F$ | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ | $S_4$ | $S_5$ | $S_6$ | $S_7$ | $S_8$ |
| Serine | 0.43 | 0.44 | 0.62 | 0.49 | 0.17 | 0.57 | 0.64 | 0.46 |
| Serine phosphate | 0.12 | 0.23 | 0.44 | 0.31 | 0.04 | 0.42 | 0.42 | 0.22 |
| Ethanolamine | 0.53–0.69 | 0.53 | 0.84 | 0.62 | 0.44 | 0.68 | 0.75 | 0.70 |
| 2-Aminoethyl phosphate | 0.46 | 0.29 | 0.55 | 0.41 | 0.06 | 0.40 | 0.46 | 0.44 |
| Lombricine | 0.55 | 0.23 | 0.31 | 0.26 | 0 | 0.18 | 0.28 | 0.23 |
| SEP | 0.34 | 0.15 | 0.22 | 0.23 | 0 | 0.13 | 0.20 | 0.19 |

# TABLE 43

## $R_F$ VALUES OF SOME INDOLE DERIVATIVES

(Ž. Procházka, V. Šanda and K. Macek, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 2928)

Solvents: $S_1$ = Petroleum ether–methanol–water. Water (50 ml), methanol (100 ml) and petroleum ether (100 ml) are placed on the tank floor. Petroleum ether (b.p. 60–70°)–methanol (25:1) is the solvent used as the mobile phase.

$S_2$ = Carbon tetrachloride–acetic acid–water. Water (50 ml), acetic acid (50 ml) and carbon tetrachloride (50 ml) are placed on the tank floor in a broad dish. The mobile phase is carbon tetrachloride–glacial acetic acid (50:1).

$S_3$ = Isopropyl ether–water. Isopropyl ether (50 ml) and water (50 ml) with the addition (in certain cases) of 0.88 ammonia (10 ml) are placed on the tank floor. The mobile phase is isopropyl ether saturated with water (or in certain cases with 2% ammonia).

$S_4$ = Butyl acetate–water. Water (50 ml) and butanol-free butyl acetate (50 ml) (butanol drastically increases the $R_F$) are placed on the floor of the tank. The mobile phase is freshly prepared butyl acetate (b.p. 126°) saturated with water.

$S_5$ = Chloroform saturated with formamide using formamide-impregnated paper.

$S_6$ = Benzene saturated with formamide using formamide-impregnated paper.

$S_7$ = Cyclohexane saturated with formamide using formamide-impregnated paper.

$S_8$ = Isopropanol–0.88 ammonia–water (10:1:1), used as a one phase system.

Paper: (a) Whatman No. 4 for water-saturated systems (ascending); equilibrated first.

(b) Whatman No. 2 for formamide-saturated systems (ascending). The paper was dipped in a 50% formamide solution (35 cm paper length).

Detection: In the case of formamide-impregnated papers, the chromatograms were developed before the evaporation of the formamide.

Formaldehyde reagent, Ehrlich reagent, Salkowski reagent, 2,4-dinitrophenylhydrazone, Jaffé reagent, together with the aid of U.V.

| *No.* | *Compound* | $R_F$ | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | $S_1$ | $S_2$ | $S_3$[j] | $S_4$ | $S_5$ | $S_6$ | $S_7$ |
| 1 | Indole | 0.38 | 0.88 | m | m | 0.82 | 0.81 | 0.24 |
| *Monosubstituted derivatives* | | | | | | | | |
| | (*a*) *Neutral* | | | | | | | |
| 2 | 3-Methylindole (Skatole) | 0.58 | 0.93 | m | m | 0.92 | 0.89 | 0.51 |
| 3 | 3-Propylindole | 0.78 | | | | | | |
| 4 | 3-Allylindole | 0.69 | | | | | | |
| 5 | 3,3′-Diindolylmethane | 0.18 | | | | 0.87 | 0.86 | 0.04 |
| 6 | 3-Hydroxymethylindole[a] | | m | | | 0.18 | | |
| 7 | 3-(2′-Hydroxyethyl)-indole (Tryptophol) | | 0.19 | 0.835 | 0.92 | 0.39 | | |
| 8 | 3-(3-Indolyl)-propanol-1 | | 0.35 | 0.875 | | | | |
| 9 | 1-(3-Indolyl)-propanol-2 | 0.034 | 0.53 | 0.87 | | | | |
| 10 | 4-(3-Indolyl)-butanol-1 | 0.055 | 0.55 | 0.925 | m | | | |
| 11 | 4-(3-Indolyl)-butanol-2 | | 0.60 | | | | | |
| 12 | Indole-3-aldehyde[h] | | 0.10 | 0.78 | 0.93 | 0.33 | | |
| 13 | 3-Indolylacetaldehyde | | 0.32 | | | | | |
| 14 | 3-Indolylacetone | 0.075 | 0.72 | 0.925 | m | 0.81 | | |
| 15 | 1-(3-Indolyl)-buten-1-one-3[i] | | 0.25 | 0.825<br>0.41 | m | | | |
| 16 | 3-(3-Indolyl)-propanediol-1,2 | | 0.02 | 0.49[k] | 0.72 | 0.05 | | |
| 17 | Ascorbigen | | | 0.04 | 0.51 | | | |

| No. | Compound | $S_1$ | $S_2$ | $S_3$[j] | $S_4$ | $S_5$ | $S_6$ | $S_7$ |
|---|---|---|---|---|---|---|---|---|
| 18 | "Substance C" | | | 0.00 | 0.12 | | | |
| 19 | 3-Indolylacetonitrile | | 0.55 | | | | | |
| 20 | 3-Indolylacetamide | | 0.05 | | 0.68 | | | |
| 21 | γ-(3-Indolyl)-butyramide | | 0.13 | | 0.85 | | | |
| 22 | 3-Indolylacetodiethylamide | | 0.88 | | | | | |
| 23 | 1-Diazo-2,3-dioxo-3-(3-indolyl)-propane | | 0.37 | | m | | | |
| 24 | 2,3-Dioxo-3-(3-indolyl)-propanol-1[h] | | 0.00 | | 0.29 | | | |
| 25 | 2-Hydroxyindole (Oxindole)[i] | 0.06 | 0.68 | 0.59 | | 0.61 | | |
| 26 | 5-Hydroxyindole | | 0.035 | 0.80 | 0.925 | 0.065 | | |
| 27 | 5-Methoxyindole | | 0.79 | | | | | |
| | *(b) Acids and esters* | | | | | | | |
| 28 | Indole-3-carboxylic acid | | 0.13 | 0.74 | 0.90 | 0.00[g] | | |
| 29 | Indole-3-carboxylic acid methyl ester | 0.09 | 0.53 | 0.94 | m | | | |
| 30 | Indole-2-carboxylic acid | 0.00 | 0.54 | | | 0.03[g] | | |
| 31 | Indole-2-carboxylic acid methyl ester | 0.53 | 0.98 | m | m | | | |
| 32 | 3-Indolylacetic acid (Heteroauxin) | 0.00 | 0.25 | 0.76 | 0.92 | 0.03[g] | | |
| 33 | 3-Indolylacetic acid methyl ester | 0.175 | 0.775 | 0.94 | m | | | |
| 34 | β-(3-Indolyl)-propionic acid | | 0.46 | 0.805 | m | | | |
| 35 | β-(3-Indolyl)-propionic acid methyl ester | 0.27 | 0.85 | 0.90 | | | | |
| 36 | γ-(3-Indolyl)-butyric acid | 0.01 | 0.715 | 0.91 | m | 0.19[g] | | |
| 37 | γ-(3-Indolyl)-butyric acid methyl ester | 0.375 | 0.92 | m | m | 0.96 | | 0.29 |
| 38 | 3-Indolylpyruvic acid[h] | | 0.04 | 0.72 | | | | |
| 39 | 3-Indolylpyruvic acid methyl ester[b] | 0.13 | 0.79 | 0.94 | | | | |
| 40 | 3-Indolylglyoxylic acid | | 0.00 | | 0.35[c] | | | |
| 41 | β-(3-Indolyl)-acrylic acid | | 0.14 | 0.695 | m | | | |
| 42 | β-(3-Indolyl)-lactic acid | | 0.02 | 0.036 | 0.76 | | | |
| 43 | β-(3-Indolyl)-lactic acid methyl ester | | 0.32 | 0.76 | | | | |

TABLE 43 *(continued)*

| No. | Compound | $R_F$ | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | $S_1$ | $S_2$ | $S_3$[j] | $S_4$ | $S_5$ | $S_6$ | $S_7$ |
| | *(b) Acids* | | | | | | | |
| 54 | 5-Hydroxy-3-indolylacetic acid | | 0.00 | | 0.56 | | | |
| 55 | β-(2-Hydroxy-3-indolyl)-propionic acid | | 0.15 | 0.88 | n | | | |

oroform

ht; 2 %
sodium

## TABLE 43

### $R_F$ VALUES OF SOME INDOLE DERIVATIVES

(Ž. PROCHÁZKA, V. ŠANDA AND K. MACEK, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 2928)

Solvents: $S_1$ = Petroleum ether–methanol–water. Water (50 ml), methanol (100 ml) and petroleum ether (100 ml) are placed on the tank floor. Petroleum ether (b.p. 60–70°)–methanol (25:1) is the solvent used as the mobile phase.
$S_2$ = Carbon tetrachloride–acetic acid–water. Water (50 ml), acetic acid (50 ml) and carbon tetrachloride (50 ml) are placed on the tank floor in a broad dish. The mobile phase is carbon tetrachloride–glacial acetic acid (50:1).
$S_3$ = Isopropyl ether–water. Isopropyl ether (50 ml) and water (50 ml) with the addition (in certain cases) of 0.88 ammonia (10 ml) are placed on the tank floor. The mobile phase is isopropyl ether saturated with water (or in certain cases with 2% ammonia).
$S_4$ = Butyl acetate–water. Water (50 ml) and butanol-free butyl acetate (50 ml) (butanol drastically increases the $R_F$) are placed on the floor of the tank. The mobile phase is freshly prepared butyl acetate (b.p. 126°) saturated with water.
$S_5$ = Chloroform saturated with formamide using formamide-impregnated paper.
$S_6$ = Benzene saturated with formamide using formamide-impregnated paper.
$S_7$ = Cyclohexane saturated with formamide using formamide-impregnated paper.
$S_8$ = Isopropanol–0.88 ammonia–water (10:1:1), used as a one phase system.

Paper: (a) Whatman No. 4 for water-saturated systems (ascending); equilibrated first.
(b) Whatman No. 2 for formamide-saturated systems (ascending). The paper was dipped in a 50% formamide solution (35 cm paper length).

Detection: In the case of formamide-impregnated papers, the chromatograms were developed before the evaporation of the formamide.
Formaldehyde reagent, Ehrlich reagent, Salkowski reagent, 2,4-dinitrophenylhydrazone, Jaffé reagent, together with the aid of U.V.

| *No.* | *Compound* | $R_F$ | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | $S_1$ | $S_2$ | $S_3$[j] | $S_4$ | $S_5$ | $S_6$ | $S_7$ |
| 1 | Indole | 0.38 | 0.88 | m | m | 0.82 | 0.81 | 0.24 |
| *Monosubstituted derivatives* | | | | | | | | |
| | (*a*) *Neutral* | | | | | | | |
| 2 | 3-Methylindole (Skatole) | 0.58 | 0.93 | m | m | 0.92 | 0.89 | 0.51 |
| 3 | 3-Propylindole | 0.78 | | | | | | |
| 4 | 3-Allylindole | 0.69 | | | | | | |
| 5 | 3,3′-Diindolylmethane | 0.18 | | | | 0.87 | 0.86 | 0.04 |
| 6 | 3-Hydroxymethylindole[a] | | m | | | 0.18 | | |
| 7 | 3-(2′-Hydroxyethyl)-indole (Tryptophol) | | 0.19 | 0.835 | 0.92 | 0.39 | | |
| 8 | 3-(3-Indolyl)-propanol-1 | | 0.35 | 0.875 | | | | |
| 9 | 1-(3-Indolyl)-propanol-2 | 0.034 | 0.53 | 0.87 | | | | |
| 10 | 4-(3-Indolyl)-butanol-1 | 0.055 | 0.55 | 0.925 | m | | | |
| 11 | 4-(3-Indolyl)-butanol-2 | | 0.60 | | | | | |
| 12 | Indole-3-aldehyde[h] | | 0.10 | 0.78 | 0.93 | 0.33 | | |
| 13 | 3-Indolylacetaldehyde | | 0.32 | | | | | |
| 14 | 3-Indolylacetone | 0.075 | 0.72 | 0.925 | m | 0.81 | | |
| 15 | 1-(3-Indolyl)-buten-1-one-3[i] | | 0.25 | 0.825 | m | | | |
| 16 | 3-(3-Indolyl)-propanediol-1,2 | | 0.02 | 0.41<br>0.49[k] | 0.72 | 0.05 | | |
| 17 | Ascorbigen | | | 0.04 | 0.51 | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 18 | "Substance C" | | | 0.00 | 0.12 | | | |
| 19 | 3-Indolylacetonitrile | | 0.55 | | | | | |
| 20 | 3-Indolylacetamide | | 0.05 | | 0.68 | | | |
| 21 | γ-(3-Indolyl)-butyramide | | 0.13 | | 0.85 | | | |
| 22 | 3-Indolylacetodiethylamide | | 0.88 | | | | | |
| 23 | 1-Diazo-2,3-dioxo-3-(3-indolyl)-propane | | 0.37 | | m | | | |
| 24 | 2,3-Dioxo-3-(3-indolyl)-propanol-1[h] | | 0.00 | | 0.29 | | | |
| 25 | 2-Hydroxyindole (Oxindole)[i] | 0.06 | 0.68 | 0.59 | | 0.61 | | |
| 26 | 5-Hydroxyindole | | 0.035 | 0.80 | 0.925 | 0.065 | | |
| 27 | 5-Methoxyindole | | 0.79 | | | | | |
| | (*b*) *Acids and esters* | | | | | | | |
| 28 | Indole-3-carboxylic acid | | 0.13 | 0.74 | 0.90 | 0.00[g] | | |
| 29 | Indole-3-carboxylic acid methyl ester | 0.09 | 0.53 | 0.94 | m | | | |
| 30 | Indole-2-carboxylic acid | 0.00 | 0.54 | | | 0.03[g] | | |
| 31 | Indole-2-carboxylic acid methyl ester | 0.53 | 0.98 | m | m | | | |
| 32 | 3-Indolylacetic acid (Heteroauxin) | 0.00 | 0.25 | 0.76 | 0.92 | 0.03[g] | | |
| 33 | 3-Indolylacetic acid methyl ester | 0.175 | 0.775 | 0.94 | m | | | |
| 34 | β-(3-Indolyl)-propionic acid | | 0.46 | 0.805 | m | | | |
| 35 | β-(3-Indolyl)-propionic acid methyl ester | 0.27 | 0.85 | 0.90 | | | | |
| 36 | γ-(3-Indolyl)-butyric acid | 0.01 | 0.715 | 0.91 | m | 0.19[g] | | |
| 37 | γ-(3-Indolyl)-butyric acid methyl ester | 0.375 | 0.92 | m | m | 0.96 | | 0.29 |
| 38 | 3-Indolylpyruvic acid[h] | | 0.04 | 0.72 | | | | |
| 39 | 3-Indolylpyruvic acid methyl ester[b] | 0.13 | 0.79 | 0.94 | | | | |
| 40 | 3-Indolylglyoxylic acid | | 0.00 | | 0.35[c] | | | |
| 41 | β-(3-Indolyl)-acrylic acid | | 0.14 | 0.695 | m | | | |
| 42 | β-(3-Indolyl)-lactic acid | | 0.02 | 0.036 | 0.76 | | | |
| 43 | β-(3-Indolyl)-lactic acid methyl ester | | 0.32 | 0.76 | | | | |
| 44 | β-(3-Indolyl)-lactic acid ethyl ester | 0.03 | 0.535 | 0.87 | | | | |
| 45 | Skatylidenemalonic acid[c] | | 0.00 | | 0.24 | | | |
| 46 | N-(3-Indolylacetyl)-aspartic acid[d] | | 0.00 | | 0.13 | | | |
| | (*c*) *Bases* | | | | | | | |
| 47 | 3-Dimethylaminomethyl-indole (Gramine) | 0.025 | | 0.94[k] | | 0.04 | | |
| 48 | 3-(2-Aminoethyl)-indole (Tryptamine) | | | 0.47[k] | | | | |
| 49 | Tryptophan | 0.00 | 0.00 | 0.00 | 0.00 | | | |
| 50 | Tryptophan methyl ester | | | 0.70[k] | | | | |
| *Disubstituted derivatives* | | | | | | | | |
| | (*a*) *Neutral* | | | | | | | |
| 51 | 1-Acetyl-3-acetonyl-indole | | 0.92 | | | 0.81 | | |
| 52 | 1-(1-Acetyl-3-indolyl)-buten-1-one-3[e] | | 0.865 | | | | | |
| 53 | 2-Hydroxytryptophan[f,i] | 0.00 | 0.00 | 0.00 | | | | |

(*Continued on p. D32*)

TABLE 43 *(continued)*

| *No.* | *Compound* | $R_F$ | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | $S_1$ | $S_2$ | $S_3$[j] | $S_4$ | $S_5$ | $S_6$ | $S_7$ |
| | (*b*) *Acids* | | | | | | | |
| 54 | 5-Hydroxy-3-indolylacetic acid | | 0.00 | | 0.56 | | | |
| 55 | β-(2-Hydroxy-3-indolyl)-propionic acid | | 0.15 | 0.88 | n | | | |
| 56 | 1-Acetyl-3-indolylacetic acid | | 0.53 | | | | | |
| 57 | 2-Carboxy-3-indolylacetic acid | | 0.03 | 0.05 | 0.735 | | | |
| 58 | β-(2-Carboxy-3-indolyl)-propionic acid | | 0.14 | 0.15 | 0.89 | | | |
| 59 | β-(2-Carboxy-3-indolyl)-propionic acid diethyl ester | 0.64 | m | m | n | | | |
| | (*c*) *Bases* | | | | | | | |
| 60 | 5-Hydroxytryptamine (Serotonin) | 0.00 | 0.00 | 0.02[k] | | 0.00 | | |
| 61 | 5-Methoxytryptamine | | | 0.25[k] | | | | |
| 62 | 6-Methoxytryptamine | | | 0.23[k] | | | | |
| *Trisubstituted derivatives* | | | | | | | | |
| 63 | 2,3-Dimethyl-5-nitroindole | 0.13 | 0.9 | | | | 0.72 | |
| 64 | 2,3-Dimethyl-7-nitroindole | 0.75 | m | | | | 0.92 | |
| 65 | 2-Methyl-6-nitroindole-3-carboxylic acid | | 0.05 | 0.015 | 0.575 | | | |
| 66 | 5,6-Dimethoxytryptamine | | | 0.025[k] | | | | |
| 67 | 2,3-Dimethyl-5-aminoindole | 0.03 | 0.00 | 0.25[k] | | | 0.14[g] | |
| 68 | 2,3-Dimethyl-7-aminoindole | 0.075 | 0.00 | 0.89[k] | | | 0.27[g] | |
| 69 | 2-Methyl-3-ethyl-5-aminoindole | | | | | 0.61[g] | 0.26[g] | |
| 70 | 2-Methyl-3-ethyl-7-aminoindole | | | | | 0.65[g] | 0.40[g] | |
| 71 | 2,3-Dimethyl-4-methoxyindole | | | | | | | 0.41 |
| 72 | 2,3-Dimethyl-5-methoxyindole | | | | | | | 0.45 |
| 73 | 2,3-Dimethyl-6-methoxyindole | | | | | | | 0.41 |
| 74 | 2,3-Dimethyl-7-methoxyindole | | | | | | | 0.81 |

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[a] $R_F$ of 0.33 in amyl ether–dil. ammonia system.

[b] Apparently not the methyl ester of 38 but a reaction product of 3-indolylpyruvic acid with diazomethane.

[c] Because of its strongly acidic character, the spot is elongated and the $R_F$ changes with concentration.

[d] The $R_F$ value was very variable.

[e] Tentative structure only.

[f] Possesses a suitable $R_F$ in butanol–acetic acid–water (4:1:5), which is, however, almost indistinguishable from that of tryptophan.

[g] A better separation of the acids and certain amino derivatives is achieved by the addition of 5% ammonium formate to the formamide.

[h] The compound gives an orange to red colour with 2,4-dinitrophenylhydrazine.

[i] The compound reacts with Jaffé reagent.

[j] The $R_F$ in this system is only considered to be of relative value.

[k] In the presence of ammonia.

[m] Compound moves with the solvent front.

[n] Compound decomposes.

## TABLE 44

### $R_F$ VALUES OF SOME NAPHTHOLS AND RELATED COMPOUNDS

(R. J. W. BYRDE, D. F. DOWNING AND D. WOODCOCK, *Biochem. J.*, 72 (1959) 344)

Solvents: $S_1$ = Aqueous 0.1 *N* ammonia.
$S_2$ = Butan-1-ol–ethanol–3 *N* ammonia (4:1:5, by vol.).
$S_3$ = Benzene–propionic acid–water (10:7:1).
Detection: 0.2% *p*-nitrobenzenediazonium fluoroborate in acetone (for $S_1$); U.V. light; 2% aqueous ferric chloride; B.D.H. universal indicator adjusted to pH 9–10 with sodium hydroxide.

| *Compound* | $R_F$ | | |
|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ |
| 2-Naphthol | 0.48 | | |
| 5-Hydroxy-2-methoxynaphthalene | 0.39 | | |
| 6-Hydroxy-2-methoxynaphthalene | 0.28 | | |
| 7-Hydroxy-2-methoxynaphthalene | 0.36 | | |
| 8-Hydroxy-2-methoxynaphthalene | 0.43 | | |
| 7-Methoxycoumarin | | 0.88 | |
| 4-Ethoxysalicylic acid | | 0.68 | |
| 4-Methoxysalicylic acid | 0.69 | 0.59 | 0.78 |

## TABLE 45

### $R_F$ VALUES OF SOME AURONES

(D. ROUBATOVÁ, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 2166)*

Solvent: Aqueous 30% acetic acid (*d* 1.038).
Paper: Schleicher & Schüll No. 20436; paper equilibrated for 12 h (descending).
Temperature of run: 19 ± 1°.
Detection: U.V. light (after exposure to ammonia vapour); antimony trichloride in chloroform (for 2-benzylidenecumaran-3-one).

| *Compound* | $R_F$ |
|---|---|
| 2-Benzylidenecumaran-3-one | 0.84 |
| 2-Benzylidene-6-hydroxycumaran-3-one | 0.40 |
| 2-Benzylidene-4,6-dihydroxycumaran-3-one | 0.25 |
| 2-Benzylidene-6,7-dihydroxycumaran-3-one | 0.15 |
| 2-(4-Methoxybenzylidene)-cumaran-3-one | 0.42 |
| 2-(4-Methoxybenzylidene)-6-hydroxycumaran-3-one | 0.22 |
| 2-(4-Methoxybenzylidene)-4,6-dihydroxycumaran-3-one | 0.12 |
| 2-(4-Methoxybenzylidene)-6,7-dihydroxycumaran-3-one | 0.18 |
| 2-(3,4-Dihydroxybenzylidene)-cumaran-3-one | 0.24 |
| 2-(3,4-Dihydroxybenzylidene)-6-hydroxycumaran-3-one | 0.10 |
| 2-(3-Methoxy-4-hydroxybenzylidene)-cumaran-3-one | 0.25 |
| 2-(3-Methoxy-4-hydroxybenzylidene)-6-hydroxycumaran-3-one | 0.12 |
| 2-(3-Methoxybenzylidene)-4,6-dihydroxycumaran-3-one | 0.03 |
| 2-(3-Methoxy-4-hydroxybenzylidene)-6,7-dihydroxycumaran-3-one | 0.09 |
| 2-(3,4-Dimethoxybenzylidene)-cumaran-3-one | 0.24 |
| 2-(3,4-Dimethoxybenzylidene)-6-hydroxycumaran-3-one | 0.10 |
| 2-(3,4-Dimethoxybenzylidene)-4,6-dihydroxycumaran-3-one | 0.04 |

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* First appeared in *Chem. listy*, 52 (1958) 1120.

## TABLE 46

### $R_F$ VALUES OF VARIOUS SUGARS AND POLYOLS

(R. VAN HEYNINGEN, *Biochem. J.*, 73 (1959) 197)

Solvents: $S_1$ = Phenol–water (100:28, w/v).
$S_2$ = Benzene–butan-1-ol–pyridine–water (1:5:3:3; upper phase).
$S_3$ = Butanol–acetic acid–water (40:9:20).
Paper: Whatman No. 1 (descending).
Detection: $AgNO_3$–NaOH reagent; aniline phthalate.

| *Compound* | $R_F$ | | |
|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ |
| Xylose | 0.46 | 0.43 | 0.32 |
| Glucose | 0.40 | 0.32 | 0.24 |
| Xylulose | 0.61 | 0.54 | 0.39 |
| Sorbitol | 0.48 | 0.29 | 0.26 |
| Xylitol | 0.61 | 0.35 | 0.31 |

## TABLE 47

### $R_F$ VALUES OF SOME AMINO-SUGARS AND THEIR N-ACETYL DERIVATIVES

(M. J. CRUMPTON, *Biochem. J.*, 72 (1959) 479)

Solvents: $S_1$ = Phenol–aq.$NH_3$ solution.
$S_2$ = *n*-Butanol–acetic acid–water (4:1:5, by vol; upper phase).
$S_3$ = *n*-Butanol–pyridine–water (6:4:3, by vol.).
Paper: Whatman No. 1.
Detection: Alkaline acetylacetone (spray), then *p*-dimethylaminobenzaldehyde hydrochloride (spray).

| *Compound* | $R_F$ | | |
|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ |
| D-Glucosaminuronic acid | 0.23 | 0.61 | 0.68 |
| D-Fructosamine | 0.62 | 1.71 | — |
| D-Allosamine | 0.66 | 1.65 | — |
| D-Galactosamine | 0.69 | 1.61 | — |
| D-Talosamine | 0.75 | 1.66 | — |
| D-Glucosamine | 0.76 | 1.53 | — |
| Muramic acid | 0.76 | 1.26 | 1.39 |
| D-Gulosamine | 0.77 | 1.61 | — |
| D-Mannosamine | 0.80 | 1.61 | — |
| D-Xylosamine | 1.00 | 1.68 | — |
| D-Fucosamine | 1.00 | 2.00 | — |
| N-Acetyl-D-galactosamine | 1.21 | 1.82 | 1.38 |
| N-Acetyl-D-glucosamine | 1.30 | 1.70 | 1.44 |
| N-Acetyl-D-allosamine | 1.32 | 1.83 | 1.54 |
| N-Acetyl-D-mannosamine | 1.36 | 1.72 | 1.62 |
| N-Acetyl-D-gulosamine | 1.42 | 1.81 | — |
| N-Acetyl-D-fructosamine | 1.44 | — | — |
| N-Acetyl-D-talosamine | 1.45 | 1.88 | 1.65 |
| N-Acetyl-D-fucosamine | 1.61 | 2.07 | 2.16 |
| N-Acetyl-D-xylosamine | 1.62 | 1.84 | 2.14 |

## TABLE 48

### $R_G$ VALUES OF SOME OLIGOSACCHARIDES

(A. R. ARCHIBALD AND D. J. MANNERS, *Biochem. J.*, 73 (1959) 292)

Solvent: Ethyl acetate–pyridine–water (10:4:3, by vol.).

| *Compound* | $R_G$* |
|---|---|
| Glucose | 1.00 |
| Maltose | 0.62 |
| Isomaltose | 0.47 |
| Maltotriose | 0.41 (0.39) |
| Panose | 0.28 |
| Maltotetrose | 0.26 |
| $6^3$-α-Glucosylmaltotriose | 0.18 |

* $R_G = R_F$ value relative to that of glucose.

## TABLE 49

### $R_F$ VALUES OF CYCLOHEXANEDIOLS AND RELATED GLUCURONIDES

(T. H. ELLIOTT, D. V. PARKE AND R. T. WILLIAMS, *Biochem. J.*, 72 (1959) 193)

Solvents: $S_1$ = Butan-2-one–xylene–water (1:1:1, by vol.).
$S_2$ = Propanol–aq. $NH_3$ soln. (sp. gr. 0.88) (7:3, v/v).
$S_3$ = Butan-1-ol–acetic acid–water (4:1:2, by vol.).
Paper: Whatman No. 4.
Detection: Ammoniacal silver nitrate (for cyclohexanediols). 2% naphthoresorcinol in 33% aqueous trichloroacetic acid, heating to 135° for 5 min after air drying (for the glucuronides).

| *Compound* | $R_F$ | | |
|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ |
| *trans*-Cyclohexane-1,2-diol | 0.25 | | |
| *cis*-Cyclohexane-1,2-diol | 0.38 | | |
| Cyclohexyl glucuronide | | 0.55 | 0.59 |
| *trans*-Cyclohexane-1,2-diol glucuronide | | 0.46 | 0.42 |
| *cis*-Cyclohexane-1,2-diol glucuronide | | 0.43 | 0.40 |

## TABLE 50

### $R_F$ VALUES OF SOME PHENYLCARBONYL COMPOUNDS

(Z. ARNOLD AND J. ŽEMLIČKA, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 2378)

Solvent: Cyclohexane (paper impregnated with dimethyl formamide).

| *Compound* | $R_F$ |
|---|---|
| α-Methyl-β-dimethylaminocinnamaldehyde | 0.1 |
| α-Methyl-β-dimethylaminovinyl phenyl ketone | 0.05 |

# TABLE 51

## $R_F$ VALUES OF SOME KETONE THIOSEMICARBAZONES

(J. FRANC, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 2096)*

Solvents: $S_1$ = Cyclohexane-*n*-butanol (25:1).
$S_2$ = Chlorocyclohexane.
$S_3$ = Carbon tetrachloride.
$S_4$ = Chlorobenzene.
Paper: Whatman No. 1 (descending).
Impregnation: (a) 30% ethanolic formamide.
(b) 20% ethanolic acetamide.
(c) 30% ethanolic dimethylformamide.
Chromatography: Carried out, after evaporation of ethanol, in a tank saturated with mobile and stationary phase vapours.
Temperature of run: 26–31°.
Detection: 5% silver nitrate spray followed by an aqueous 1–2% ammonia solution; Ehrlich reagent and U.V. light.

| *Ketone* | $R_F$ | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $S_1$ | | | $S_2$ | | | $S_3$ | | $S_4$ | |
| | *a* | *b* | *c* | *a* | *b* | *c* | *a*[a] | *c* | *a*[b] | *c*[b] |
| Cyclohexanone | 0.05 | 0.10 | 0.14 | 0.12 | 0.33 | 0.53 | 0.12 | 0.42 | 0.32 | 0.79 |
| 2-Methylcyclohexanone | 0.13 | 0.23 | 0.22 | 0.30 | 0.54 | 0.66 | — | 0.51 | 0.60 | 0.84 |
| 3-Methylcyclohexanone | 0.11 | 0.20 | 0.20 | 0.29 | 0.50 | 0.62 | 0.30 | 0.51 | 0.57 | 0.83 |
| 4-Methylcyclohexanone | 0.10 | 0.18 | 0.19 | 0.27 | 0.48 | 0.60 | 0.28 | 0.50 | 0.55 | 0.83 |
| Cyclopentanone | 0.03 | 0.06 | 0.08 | 0.07 | 0.20 | 0.41 | 0.08 | 0.37 | 0.23 | 0.74 |
| 2-Methylpentanone | 0.07 | 0.13 | 0.15 | 0.21 | 0.45 | 0.54 | 0.22 | 0.48 | 0.47 | 0.80 |
| 3-Methylpentanone | 0.06 | 0.10 | 0.12 | 0.16 | 0.38 | 0.52 | 0.19 | 0.46 | 0.43 | 0.79 |
| Dimethyl ketone | 0.016 | 0.03 | 0.04 | 0.035 | 0.09 | 0.21 | 0.02 | 0.26 | 0.08 | 0.59 |
| Methyl ethyl ketone | 0.03 | 0.05 | 0.09 | 0.09 | 0.22 | 0.26 | 0.08 | 0.42 | 0.22 | 0.71 |
| Diethyl ketone | — | — | — | — | 0.49 | — | 0.22 | 0.56 | 0.47 | 0.79 |
| Ethyl propyl ketone | 0.21 | 0.25 | 0.30 | 0.40 | 0.63 | 0.73 | 0.37 | 0.59 | 0.70 | 0.84 |
| Methyl butyl ketone | 0.18 | 0.18 | 0.25 | 0.40 | 0.60 | 0.66 | 0.41 | 0.59 | 0.70 | 0.82 |
| Methyl isobutyl ketone | 0.18 | 0.19 | 0.26 | 0.39 | 0.59 | 0.68 | 0.33 | 0.59 | 0.63 | 0.82 |
| Methyl amyl ketone | 0.32 | 0.35 | — | — | 0.80 | — | 0.61 | — | 0.84 | 0.85 |

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* First appeared in *Chem. listy*, 52 (1958) 2311.
[a] Chromatographed at 31°; [b] chromatographed at 30°; others at 27° ± 1°.

## TABLE 52

### $R_F$ VALUES OF SOME NITRODERIVATIVES OF AROMATIC HYDROCARBONS

(J. FRANC AND J. KNIŽEK, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 2299)

Solvent: Stationary phase, liquid paraffin; mobile phase, ethanol–acetic acid–water (100:5:70).
Paper: Whatman No. 1 (descending) impregnated by dipping in a 20% petroleum ether solution of liquid paraffin (b.p. 190–275°) and allowing the petroleum ether to evaporate for quarter of an hour.
Length of run: 20–25 cm.
Detection: 10% $SnCl_2$ solution (reduction), followed by Ehrlich reagent.

| *Compound* | $R_F$ | *Compound* | $R_F$ |
|---|---|---|---|
| 1,3-Dinitrobenzene | 0.58 | 2,4-Dinitro-*m*-xylene | 0.37 |
| 1,3,5-Trinitrobenzene | 0.45 | 2,3-Dinitro-*p*-xylene | 0.62 |
| 2,3-Dinitrotoluene | 0.73 | 2,5-Dinitro-*p*-xylene | 0.33 |
| 2,6-Dinitrotoluene | 0.55 | 2,6-Dinitro-*p*-xylene | 0.38 |
| 2,5-Dinitrotoluene | 0.44 | 2,4-Dinitroethylbenzene | 0.39 |
| 3,4-Dinitrotoluene | 0.72 | 2,6-Dinitroethylbenzene | 0.47 |
| 3,5-Dinitrotoluene | 0.45 | 3,4,6-Trinitro-*o*-xylene | 0.50 |
| 2,4-Dinitrotoluene | 0.52 | 3,4,5-Trinitro-*o*-xylene | 0.65 |
| 2,4,6-Trinitrotoluene | 0.36 | 2,4,6-Trinitro-*m*-xylene | 0.25 |
| 3,5-Dinitro-*o*-xylene | 0.41 | 4,5,6-Trinitro-*m*-xylene | 0.82 |
| 3,4-Dinitro-*o*-xylene | 0.74 | 2,3,5-Trinitro-*p*-xylene | 0.43 |
| 4,5-Dinitro-*o*-xylene | 0.71 | 2,4,6-Trinitroethylbenzene | 0.27 |
| 3,6-Dinitro *o*-xylene | 0.31 | Dinitromesitylene | 0.32 |
| 4,6-Dinitro-*o*-xylene | 0.43 | | |

## TABLE 53

### $R_F$ VALUES OF SOME 2,4-DINITROPHENYLHYDRAZONES OF HOMOLOGOUS CYCLIC KETONES

(V. DUDEK AND J. STUCHLÍK, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 3797)

Solvents: $S_1$ = Methanol–water–acetic acid (90:10:3).
$S_2$ = Methanol–water–acetic acid (95:5:3).
$S_3$ = 80% methanol.
$S_4$ = Methanol–water–acetic acid (80:20:2).
Stationary phase: Liquid paraffin (b.p. 200–220°) in petroleum ether (b.p. 30–45°) for impregnation.
Temperature of run: 20°.
Paper: Whatman No. 1 ($S_1$, $S_2$ descending; $S_3$, $S_4$ ascending).
Detection: Red light; U.V.; 10% KOH.

| *2,4-Dinitrophenylhydrazone of* | $R_F$ | | | |
|---|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ | $S_4$ |
| Cyclopentanone | 0.45 | 0.53 | — | — |
| Cyclohexanone | 0.42 | 0.50 | 0.93 | 0.95 |
| Cycloheptanone | 0.33 | 0.44 | 0.75 | 0.86 |
| Cyclooctanone | 0.29 | 0.41 | 0.67 | 0.80 |
| Cyclononanone | 0.26 | 0.37 | 0.56 | 0.72 |
| Cyclodecanone | 0.20 | 0.33 | — | — |
| Cyclododecanone | 0.13 | 0.25 | — | — |

## TABLE 54

### $R_F$ VALUES OF SOME ALDEHYDE–RHODANINE CONDENSATION PRODUCTS

(J. FRANC, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 2102)

Solvents: $S_1$ = Chlorocyclohexane.
$S_2$ = Chlorobenzene.
$S_3$ = Carbon tetrachloride.
$S_4$ = Cyclohexane–*n*-butanol (25:1).
Paper: Whatman No. 1 (descending).
Impregnation: (a) 30% ethanolic formamide.
(b) 30% ethanolic dimethyl formamide.
Temperature of run: 21° ± 1°.
Detection: U.V. and visible light; 5% ammoniacal silver nitrate (spray).

| *Aldehyde* | $R_F$ | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $S_1$ | | $S_2$ | | $S_3$ | | $S_4$ | |
| | *a* | *b* | *a* | *b* | *a* | *b* | *a* | *b* |
| Benzaldehyde | 0.43 | 0.59 | 0.65 | 0.78 | 0.19 | 0.54 | 0.19 | 0.12 |
| 3-Hydroxybenzaldehyde | 0.014 | 0.20 | 0.017 | 0.64 | 0.00 | 0.16 | 0.15 | 0.013 |
| 3-Methoxy-4-hydroxybenzaldehyde | 0.028 | 0.20 | 0.074 | 0.69 | 0.016 | 0.10 | 0.014 | 0.011 |
| 3-Methoxy-4-benzyloxybenzaldehyde | 0.68 | 0.71 | 0.89 | 0.85 | 0.59 | 0.76 | 0.15 | 0.049 |
| 2-Hydroxy-3-methoxybenzaldehyde | 0.057 | 0.30 | 0.18 | 0.72 | 0.025 | 0.20 | 0.014 | 0.011 |
| 2-Hydroxy-3-ethoxybenzaldehyde | 0.03 | 0.16 | 0.077 | 0.67 | 0.013 | 0.12 | 0.00 | 0.07 |
| 3-Hydroxy-4-methoxybenzaldehyde | 0.067 | 0.35 | 0.19 | 0.79 | 0.075 | 0.25 | 0.027 | 0.015 |
| 3,4-Dimethoxybenzaldehyde | 0.17 | 0.47 | 0.54 | 0.91 | 0.105 | 0.34 | 0.038 | 0.017 |
| 2-Benzyloxybenzaldehyde | 0.80 | 0.75 | 0.90 | 0.86 | 0.71 | 0.77 | 0.09 | 0.08 |
| *p*-Aminobenzaldehyde | 0.037 | 0.28 | 0.06 | 0.72 | 0.16 | 0.19 | 0.00 | 0.00 |
| *p*-Nitrobenzaldehyde | 0.12 | — | 0.36 | 0.69 | 0.05 | 0.23 | 0.00 | 0.017 |
| *m*-Nitrobenzaldehyde | 0.09 | 0.37 | — | 0.68 | 0.042 | 0.23 | 0.013 | 0.022 |
| *o*-Nitrobenzaldehyde | 0.03 | 0.21 | — | 0.66 | 0.015 | 0.14 | 0.00 | 0.012 |
| *p*-Chlorobenzaldehyde | 0.51 | 0.62 | 0.77 | 0.80 | 0.47 | 0.55 | 0.31 | 0.14 |
| Salicylaldehyde | 1.00 | 0.92 | — | 0.90 | — | 0.86 | — | 0.28 |
| Fural | 0.33 | 0.53 | 0.49 | 0.77 | 0.25 | 0.41 | 0.14 | 0.061 |
| *p*-Tolualdehyde | 0.67 | 0.74 | 0.82 | 0.86 | — | 0.67 | 0.425 | 0.21 |
| Rhodanine | 0.03 | 0.37 | 0.00 | 0.63 | 0.00 | 0.24 | 0.00 | 0.058 |

## TABLE 55

### $R_F$ VALUES OF SOME ALKYL-BENZIDINE DERIVATIVES

(M. MATRKA, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 294)

Solvent: Cyclohexane.
Paper: Whatman No. 4 (descending).
Impregnation: 25% dimethylformamide.
Detection: 0.1 *N* cerium (IV) sulphate solution (spray).

| *Compound* | $R_F$ |
|---|---|
| N,N,N′,N′-Tetraethylbenzidine | 0.92 |
| N,N,N′-Triethylbenzidine | 0.75 |
| N,N′-Diethylbenzidine | 0.15 |

## TABLE 56

### $R_F$ VALUES OF KETONE–RHODANINE CONDENSATION PRODUCTS

(J. FRANC, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 2102)

Solvents: $S_1$ = Chlorocyclohexane.
$S_2$ = Carbon tetrachloride.
$S_3$ = Chlorobenzene.
$S_4$ = Cyclohexane–*n*-butanol (25:1).
Paper: Whatman No. 1 (descending).
Impregnation: (a) 30 % ethanolic formamide.
(b) 30 % ethanolic dimethyl formamide.
Temperature of run: 20° ± 1°.
Detection: U.V. light or in visible light.

| *Ketone* | $R_F$ | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $S_1$ | | $S_2$ | | $S_3$ | | $S_4$ | |
| | *a* | *b* | *a* | *b* | *a* | *b* | *a* | *b* |
| Acetone | 0.33 | 0.74 | 0.22 | 0.81 | 0.54 | 0.92 | 0.26 | 0.32 |
| Methyl ethyl ketone | 0.61 | 0.84 | 0.50 | 0.88 | 0.79 | 0.92 | 0.57 | 0.47 |
| Ethyl propyl ketone | 0.90 | 0.93 | 0.85 | 0.935 | 0.92 | 0.93 | 0.88 | 0.76 |
| Diethyl ketone | 0.80 | 0.91 | 0.71 | 0.92 | 0.91 | 0.89 | 0.77 | 0.63 |
| Methyl isobutyl ketone | 0.87 | 0.92 | 0.82 | 0.94 | 0.925 | 0.91 | 0.85 | 0.74 |
| Methyl butyl ketone | 0.89 | 0.93 | 0.85 | 0.94 | 0.925 | 0.93 | 0.87 | 0.75 |
| Cyclopentanone | 0.52 | 0.83 | 0.43 | 0.89 | 0.75 | 0.88 | 0.49 | 0.43 |
| 2-Methylcyclopentanone | 0.72 | 0.88 | 0.71 | 0.90 | 0.87 | 0.93 | 0.62 | 0.65 |
| 3-Methylcyclopentanone | 0.70 | 0.88 | 0.68 | 0.89 | 0.87 | 0.92 | 0.61 | 0.65 |
| x,x'-Dimethylcyclopentanone | 0.72 | 0.87 | 0.70 | 0.89 | 0.87 | 0.93 | 0.615 | 0.64 |
| Cyclohexanone | 0.78 | 0.89 | 0.75 | 0.90 | 0.88 | 0.935 | 0.62 | 0.66 |
| 2-Methylcyclohexanone | 0.89 | 0.92 | 0.86 | 0.92 | 0.93 | 0.935 | 0.785 | 0.75 |
| 3-Methylcyclohexanone | 0.88 | 0.925 | 0.85 | 0.92 | 0.93 | 0.925 | 0.785 | 0.75 |
| 4-Methylcyclohexanone | 0.87 | 0.92 | 0.84 | 0.915 | 0.93 | 0.915 | 0.77 | 0.73 |
| Rhodanine | 0.00 | 0.18 | 0.00 | 0.22 | 0.04 | 0.60 | 0.03 | 0.06 |

## TABLE 57

### $R_F$ VALUES OF 2,4,5-TRIHYDROXYBUTYROPHENONE AND RELATED COMPOUNDS

(B. D. ASTILL, D. W. FASSETT AND R. L. ROUDABUSH, *Biochem. J.*, 72 (1959) 451)

Solvents: $S_1$ = Organic phase of freshly mixed *n*-butanol–acetic acid–water (4:1:5, by vol.).
$S_2$ = Organic phase of benzene–acetic acid–water (2:2:1, by vol.).
Paper: Whatman No. 1.
Detection: A: Aqueous 1 % (w/v) $AgNO_3$ soln.–aq. 10 % $NH_3$ soln. (1:1).
B: 1 % $FeCl_3$ in aq. 50 % v/v ethanol.
C: Freshly mixed 1 % (w/v) sulphanilic acid in 3 *N* HCl–aq. 5 % (w/v) $NaNO_2$ (1:1, v/v). Dried paper suspended in $NH_3$ atmosphere.
D: U.V.

| *Compound* | $R_F$ | |
|---|---|---|
| | $S_1$ | $S_2$ |
| 2,4,5-Trihydroxybutyrophenone | 0.92–0.95 | 0.42–0.44 |
| 4-Butyryl-2,5-dihydroxyphenyl glucosiduronic acid | 0.75–0.81 | 0.14 |
| 5-Butyryl-2,4-dihydroxyphenyl hydrogen sulphate | 0.68–0.72 | 0.12 |
| Probably 5-O-glucuronide of 2,4,5-trihydroxybutyrophenone | 0.45–0.55 | 0.05 |

## TABLE 58

### $R_F$ VALUES OF CYSTEINE AND N,S-DIACETYL-CYSTEINE

(J. F. BERRY AND V. P. WHITTAKER, *Biochem. J.*, 73 (1959) 447)

Solvent: *n*-Butanol saturated with water.
Paper: Whatman No. 1 (ascending).
Detection: Ninhydrin; hydroxylamine and FeCl$_3$.

| *Compound* | $R_F$ |
|---|---|
| Cysteine | 0.00 |
| N,S-Diacetylcysteine | 0.12 |

## TABLE 59

### $R_F$ VALUES OF SOME PEPTIDES

(B. KEIL AND F. ŠORM, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 1558)[*]

Solvent: *n*-Butanol–acetic acid–water (144:13:43).
Paper: Whatman No. 4 (descending).
Detection: 0.5% ninhydrin in acetone (dip).
$R_F$-glu = $R_F$ relative to that of glutamic acid (run under the same conditions).

| *Peptide* | $R_F$*-glu* | *Peptide* | $R_F$*-glu* |
|---|---|---|---|
| CySO$_3$H | 0.1 | (Leu,Val,CySO$_3$H) | 1.9 |
| (Ser,CySO$_3$H) | 0.2 | (CySO$_3$H,Ala) | 0.7 |
| Thr·CySO$_3$H | 0.3 | (Thr,CySO$_3$H,Val) | 1.2 |
| (Ser,Thr,CySO$_3$H) | 0.05 | CySO$_3$H·(Lys,Asp) | 0.1 |
| (Ser,CySO$_3$H,MetSO$_2$) | | (CySO$_3$H,Lys) | 0.1 |
| (CySO$_3$H,MetSO$_2$) | 0.2 | Asp | 0.7 |
| (Gly,CySO$_3$H) | 1.0 | CySO$_3$H·Lys | 0.1 |
| (Ileu,CySO$_3$H,Ala) | 1.0 | | |

## TABLE 60

### RELATIVE PAPER ELECTROPHORETIC MOBILITIES OF CYSTEIC ACID PEPTIDES

(B. KEIL AND F. ŠORM, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 1558)[*]

Electrolyte: 4 ml pyridine + 1 ml acetic acid diluted to 1 litre with distilled water (pH 5.6).
Potential: 1400 V (*i.e.* 30 V/cm).
Paper: Whatman No. 1.
Type: Vertical; anode connected to upper trough.
Migration units: Relative to glutamic acid (glutamic acid = 1).
Detection: Ninhydrin.

| *Peptide* | *Relative mobility* | *Peptide* | *Relative mobility* |
|---|---|---|---|
| Ser·CySO$_3$H | 1.0 | CySO$_3$H·Val | 0.9 |
| Phe·CySO$_3$H | 1.0 | CySO$_3$H·MetSO$_2$ | 0.9 |
| Ileu·CySO$_3$H | 0.85 | Leu·Val·CySO$_3$H | 0.6 |
| Thr·CySO$_3$H | 0.95 | Ser·CySO$_3$H·MetSO$_2$ | 0.7 |
| CySO$_3$·Ala | 1.0 | Ileu·CySO$_3$H·Ala | 0.7 |
| CySO$_3$·Gly | 1.05 | Thr·CySO$_3$H·Val | 0.65 |

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[*] First appeared in *Chem. listy*, 52 (1958) 1327.

## TABLE 61

### $R_F$ VALUES OF SOME MERCAPTURIC ACIDS, S-SUBSTITUTED CYSTEINES

(H. G. BRAY, T. J. FRANKLIN AND S. P. JAMES, *Biochem. J.*, 73 (1959) 465)

Solvents: $S_1$ = Butan-1-ol–acetic acid–butyl acetate (24:10:5:2, v/v).
$S_2$ = Butan-1-ol saturated with aqueous 2 *N* $NH_3$ solution.
$S_3$ = Benzene–acetic acid–water (2:2:1, v/v).
Paper: Whatman No. 4 or 3MM (descending).
Time of run: *T* (hours).
Detection: Potassium dichromate–silver nitrate reagent; ninhydrin.

| *Compound* | $R_F$ | | | | | |
|---|---|---|---|---|---|---|
| | $S_1$ | *T* | $S_2$ | *T* | $S_3$ | *T* |
| S-Butyl-L-cysteine | 0.73 | 7 | 0.35 | 16 | — | |
| N-Acetyl-S-butyl-L-cysteine | 0.92 | 7 | 0.46 | 16 | — | |
| N-Acetyl-S-(2,3,5,6-tetrachlorophenyl)-L-cysteine | 0.95 | 14 | 0.60 | 16 | — | |
| S-(2,3,5,6-Tetrachlorophenyl)-L-cysteine | 0.60* | 14 | 0.44 | 16 | — | |
| 2,3,5,6-Tetrachlorothiophenol** | — | | 0.75 | 4 | 1.0 | 4 |
| S-(2-Chloro-4-nitrophenyl)-L-cysteine | 0.50 | 16 | *** | | — | |
| S-Benzyl-L-cysteine | 0.63 | 7 | 0.22 | 16 | — | |
| N-Acetyl-S-benzyl-L-cysteine | 0.93 | 7 | 0.41 | 16 | — | |

* Streaks.
** Ascending.
*** Decomposes in this solvent.

## TABLE 62

### ELECTROPHORETIC MOBILITIES OF ARGININE AND CANAVANINE

(M. H. RICHMOND, *Biochem. J.*, 73 (1959) 261)

Electrolytes: $E_1$ = Ammonium carbonate buffer (2%, w/v), pH 9.1 (pH adjusted with 0.88 $NH_3$ soln.).
$E_2$ = Pyridine–acetic acid–water (10:0.4:90, v/v), pH 6.5.
$E_3$ = Pyridine–acetic acid–water (1:10:89, v/v), pH 3.5.
(All buffers removed by heating at 50° for 30 min).
Detection: Ninhydrin; alkaline α-naphthol–diacetyl reagent.
Paper: Whatman No. 3.
Potential gradient: 40 V/cm.
Apparatus: Kunkel (1954); closed strip method.
Units: cm (distance moved in 2 h at a potential difference of 40 V/cm).

| *Compound* | *Mobility* | | |
|---|---|---|---|
| | $E_1$ | $E_2$ | $E_3$ |
| Arginine | + 5.3 | + 31 | + 43 |
| Canavanine | — 6.7 | + 27.5 | + 43 |

+ Migration towards cathode.
— Migration towards anode.

## TABLE 63

### $R_F$ VALUES OF SOME PEPTIDE DERIVATIVES

(H. NEUMANN, Y. LEVIN, A. BERGER AND E. KATCHALSKI, *Biochem. J.*, 73 (1959) 33)

Solvents: $S_1$ = *n*-Butanol–acetic acid–water (25:6:25, v/v).
$S_2$ = *n*-Propanol–water–conc. $NH_3$ solution (100:50:1, v/v).
Paper: Whatman No. 1.
Detection: Ninhydrin; *p*-dimethylaminobenzaldehyde; diazotized sulphanilic acid; U.V. (where necessary paper chromatogram exposed to HBr gas to allow detection).
Abbreviations: Tyr = L-tyrosyl residue.
Glu = L-glutamyl residue.
Phe = L-phenylalanyl residue.
Am = amide group.
PAB = *p*-aminobenzoyl group.

| *Compound* | $R_F$ | |
|---|---|---|
| | $S_1$ | $S_2$ |
| Tyr | 0.45 | |
| $Tyr_2$ | 0.68 | |
| PAB·Tyr | | 0.76 |
| PAB·$Tyr_2$ | | 0.83 |
| *p*-Aminobenzoic acid | | 0.51 |
| Glu·Tyr | 0.52 | |
| Phe·Tyr | 0.80 | |
| Tyr·Am | 0.45 | 0.79 |
| Phe | 0.65 | |

## TABLE 64

### ELECTROPHORETIC MOBILITIES OF SOME N-BENZYLOXYCARBONYL-PEPTIDES

(H. NEUMANN, Y. LEVIN, A. BERGER AND E. KATCHALSKI, *Biochem. J.*, 73 (1959) 33)

Electrolyte: phosphate buffer, pH 6.8 (0.05 *M* $Na_2HPO_4$ and 0.05 *M* $NaH_2PO_4$).
Paper: Whatman No. 1.
Potential: 10 V/cm.
Time of run: 2 h.
Apparatus: Consden and Stanier (1952).
Units: cm.
Detection: Ninhydrin; diazotized sulphanilic acid; *p*-dimethylaminobenzaldehyde; U.V.
Abbreviations: Z = benzyloxycarbonyl group.
Tyr = L-tyrosyl residue.
Glu = L-glutamyl residue.

| *Compound* | *Mobility cm/2 h* |
|---|---|
| Tyr | — 0.4 |
| $Tyr_2$ | + 1.2 |
| Z-Tyr | + 4.5 |
| Z-$Tyr_2$ | + 3.1 |
| Z-Glu.Tyr | + 6.5 |
| Z-Glu.$Tyr_2$ | + 5.0 |
| Z-Glu.$Tyr_3$ | + 4.0 |
| Z-Glu | + 9.5 |

+ Towards anode. — Towards cathode.

## TABLE 65

### $R_F$ VALUES OF SOME PEPTIDE ETHYL ESTERS, THEIR CONSTITUENT AMINO ACIDS AND SOME CARBOBENZOXY DERIVATIVES

(Z. A. SHABAROVA, N. I. SOKOLOVA AND M. A. PROKOFIEV, *Compt. rend. acad. sci. U.R.S.S.*, 128 (1959) 740)

Solvents: $S_1$ = *n*-Butanol saturated with water.
$S_2$ = *n*-Butanol–acetic acid–water (4:1:5, organic phase).
$S_3$ = Isoamyl alcohol–disodium hydrogen phosphate.
$S_4$ = *n*-Butanol saturated with $NH_3$.
Detection: Ninhydrin; U.V. light.

| *Compound* | $R_F$ | | | |
|---|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ | $S_4$ |
| *Ethyl esters of* | | | | |
| Phenylalanylglycine | | 0.73 | | |
| Leucylglycine | | 0.76 | | |
| Valylphenylalanylglycine | | 0.65 | | |
| *Amino acid components* | | | | |
| Phenylalanine | | 0.50 | — | — |
| Glycine | | 0.16 | — | 0.33 |
| Leucine | | 0.59 | | |
| Valine | | 0.42 | | |
| *Carbobenzoxy derivatives of O-aminoacyl derivatives of adenosine* | | | | |
| 2(3),5-Di-O-Cbz-phenylalanyladenosine | 0.87 | | | |
| 5-O-Cbz-phenylalanyl-2,3-isopropylideneadenosine | 0.90 | | | |
| *Other compounds* | | | | |
| Adenosine | 0.27 | | 0.55 | |
| Cytidine | | | 0.75 | |
| DNP-phenylalanine | | | — | 0.79 |

## TABLE 66

### ELECTROPHORETIC MOBILITIES OF SOME *p*-AMINOBENZOYL-PEPTIDES

(H. NEUMANN, Y. LEVIN, A. BERGER AND E. KATCHALSKI, *Biochem. J.*, 73 (1959) 33)

Electrolyte: Phosphate buffer, pH 6.8 (0.05 *M* $NaH_2PO_4$ and 0.05 *M* $Na_2HPO_4$).
Paper: Whatman No. 1.
Potential: 10 V/cm.
Time of run: 2 h.
Apparatus: Consden and Stanier (1952).
Units: cm.
Detection: Sulphanilic acid; *p*-dimethylaminobenzaldehyde; U.V.

| *Peptide* | *Mobility (cm/2 h)* |
|---|---|
| N-*p*-Aminobenzoyl-L-tyrosyl-L-tyrosine | — 7.5 |
| N-*p*-Aminobenzoyl-L-tyrosine | — 5.3 |
| *p*-Aminobenzoic acid | — 11.0 |

— Towards cathode.

## TABLE 67

### $R_F$ VALUES OF SOME AMINO ACIDS AND RELATED COMPOUNDS

(C. E. Rowe, *Biochem. J.*, 73 (1959) 438)

Solvents: $S_1$ = *n*-Butanol–acetic acid–water (63:10:27, v/v).
$S_2$ = Phenol–water (5:2, v/v).
Paper: Whatman No. 1.
Detection: Ninhydrin; phosphomolybdic acid followed by $SnCl_2$ reduction.

| *Compound* | $R_F$ | |
|---|---|---|
| | $S_1$ | $S_2$ |
| Ethanolamine | 0.20 | 0.91–0.92 |
| Serine | 0.10 | 0.40 |
| Serine methyl ester | 0.20 | 0.91 |
| Choline | 0.47 | 0.82 |
| Methoxycholine* | 0.24–0.26 | 0.89–0.90 |

* Tentative identification.

## TABLE 68

### $R_F$ VALUES OF SOME CHOLINE ESTERS

(J. F. Berry and V. P. Whittaker, *Biochem. J.*, 73 (1959) 447)

Solvent: *n*-Butanol saturated with water.
Paper: Whatman No. 1.
Detection: Iodine vapour; bromocresol purple; hydroxylamine and $FeCl_3$.

| *Compound* | $R_F$ |
|---|---|
| Choline | 0.08 |
| Acetylcholine | 0.10 |
| Propionylcholine | 0.22 |
| *n*-Butyrylcholine | 0.28 |
| *n*-Valerylcholine | 0.31 |
| Hexanoylcholine | 0.36 |
| Palmitoylcholine* | 0.70 |
| Palmitic acid* | 1.0 |

* Form a complex if run together.

## TABLE 69

### $R_F$ VALUE OF 2-(PHENYLCYCLOHEXYLACETOXY)-ETHYL-DIMETHYL-[$^{35}$S]SULPHONIUM IODIDE ($^{35}$S-THIOSPASMIN)

(O. Piták and J. Zikmund, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 4053)

Solvent: *n*-Butanol–acetic acid–water (4:1:5).
Paper: Whatman No. 1.
Detection: Dragendorff reagent; autoradiographically by FOMA-Röntgen-X-Film.

| *Compound* | $R_F$ |
|---|---|
| $^{35}$S-Thiospasmin | 0.82 |

## TABLE 70

### $R_F$ VALUES OF SOME ETHANOLAMINES

(J. FRANC AND M. HÁJKOVÁ, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 4043)

Solvents: $S_1$ = Dimethyl formamide–ethanol (3:7).
$S_2$ = *n*-Butanol–amyl alcohol (10:4).
$S_3$ = Hexanol–*n*-butanol–$NH_4OH$ (1:3:4).
Paper: Whatman No. 1 (24 × 47 cm).
Time: 5 h (at 20°).
Length of run: 25 cm (at 20°).
Temperature: $S_1$, 24°; $S_2$, 20°; $S_3$, 20°.
Detection: Gibbs reagent in *m*-cresol (0.5 g 2,6-dibromoquinone chloroimine and 0.3 g phenol in 100 ml ethanol) or 0.2–0.3% Pyrocatechol Violet.

| *Compound* | $R_F$ | | |
|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ |
| Hydroxylamine | 0.36 | — | — |
| Monoethanolamine | 0.52 | 0.32 | 0.30 |
| Diethanolamine | 0.70 | 0.47 | 0.45 |
| Triethanolamine | 0.81 | 0.62 | 0.60 |

## TABLE 71

### $R_F$ VALUES OF SOME HALOGENATED ALIPHATIC ACIDS

(C. RAPPE AND N. HELLSTRÖM, *Arkiv Kemi*, 13 (1959) 485)

Solvent: Benzene–cyclohexane mixture (60 ml + 30 ml) saturated with water, excess water removed, organic phase then filtered through paper moistened with the organic mixture. Acetic acid (10 ml) was added to this.
Time of run: 2 h (approx).
Length of run: 20–22 cm.
Paper: Whatman No. 1 (descending).
Detection: 0.04% ethanolic bromophenol blue or bromocresol green solution (spray).

| *Acid* | $R_F$ | *Acid* | $R_F$ |
|---|---|---|---|
| $CH_2ClCOOH$ | 0.23 | $CH_2ICH_2COOH$ | 0.78 |
| $CH_2BrCOOH$ | 0.30 | $CH_2BrCHBrCOOH$ | 0.71 |
| $CH_2ICOOH$ | 0.45 | $CH_3CH_2CHBrCOOH$ | 0.84 |
| $CHCl_2COOH$ | 0.15 | $CH_3CH_2CH_2CHBrCOOH$ | 0.90 |
| $CCl_3COOH$ | 0.05–0.12 | $(CH_3)_2CHCHBrCOOH$ | 0.89 |
| $CF_3COOH$ | 0 | $CH_3CH_2CH_2CH_2CHBrCOOH$ | 0.92 |
| $CH_3CHClCOOH$ | 0.59 | $CH_3COCH_2CH_2COOH$ | 0.22 |
| $CH_2ClCH_2COOH$ | 0.61 | $CH_3COCHClCH_2COOH$ | 0.44 |
| $CH_3CHBrCOOH$ | 0.69 | $CH_3COCHBrCH_2COOH$ | 0.54 |
| $CH_2BrCH_2COOH$ | 0.67 | $CH_2ClCOCH_2CH_2COOH$ | 0.30 |
| $CH_3CHICOOH$ | 0.79 | $CH_2BrCOCH_2CH_2COOH$ | 0.38 |
| | | $CH_2BrCOCHBrCH_2COOH$ | 0.62 |

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